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GAP JUNCTIONS AND CELL GROWTH: INHIBITION OF CONNEXIN43 SYNTHESIS BY ANTISENSE RNA IN GLIOMA CELLS

by

MADHU VRUT SINGH ©

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Doctor of Philosophy

Department of Biological Sciences

Edmonton, Alberta
Fall 1995

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled ***Gap Junctions and Cell Growth: Inhibition of Connexin43 Synthesis by Antisense RNA in Glioma Cells*** submitted by ***Madhu Vrut Singh*** in partial fulfillment of the requirements for the degree of ***Doctor of Philosophy.***

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To my wife Minati,
and my daughter Munmun.

ABSTRACT

Loss of cell-to-cell contacts (gap junctions) is thought to be a feature of tumorigenesis. In this study, gap junctional characteristics of the 9L cells were compared with those of the rat glioma C6 cells, and the effect of gap junctional communication on the growth rate of rat glioma cell lines *in vitro* was examined by inhibiting the expression of gap junction protein, connexin43, using antisense RNA. The 9L rat glioma cell line expresses only connexin43 in cultures. Gap junctions were more frequent between 9L cells, but rare between C6 cells by electron microscopy. Northern blot analyses showed a six-fold higher level of connexin43 mRNA in 9L cells than in C6 cells. The results from immunofluorescent staining and immunoblotting reflected these differences in the connexin43 contents of 9L and C6 cells. Dye-transfer experiments revealed that 9L cells were more extensively dye-coupled than C6 cells. Despite these differences in gap junctions, the two cell lines showed similar growth rates.

Synthesis of connexin43 in 9L glioma cells was inhibited by transfection with an antisense RNA coding plasmid. Southern blot analyses provided evidence for transfection. The expression of antisense RNA, which was under the control of a mouse metallothionein promoter, was induced by the addition of zinc acetate. Induction of antisense connexin RNA abolished almost all the immunofluorescent staining of connexin43 in 24 hours. Connexin43 protein decreased to undetectable levels on immunoblots of transfected cells after zinc treatment. Ribonuclease protection assays showed the presence of antisense RNA in the transfected cells. The growth of zinc-treated cells was decreased in both untransfected and transfected cells; however the decrease was significantly greater in the transfected cells. Also, the transfected cells grew much slower than the untransfected cells even without zinc treatment.

These results show that (a) gap junctions may affect the cell growth in 9L rat glioma but are not the major growth determining factor, (b) the antisense RNA method can be used to inhibit gap junction formation, and (c) the 9L cells provide a valuable model for studying the regulation of gap junctions in astroglia. This is the first demonstration of a direct study on the effect of inhibition of connexin43 on the growth rate of a tumor cell line.

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TABLE OF CONTENTS

ABSTRACT

ACKNOWLEDGEMENT

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

CHAPTER 1

INTRODUCTION 1

Objectives 4

CHAPTER 2

REVIEW OF LITERATURE 6

Gap junctions and their historical perspective 6

Molecular constituents of gap junctions 8

Tissue distribution of gap junctions 16

Regulation of gap junctions 22

Functions of gap junctions 27

Cell cycle and factors affecting cell proliferation 32

CHAPTER 3

MATERIALS AND METHODS 35

Plasmids 35

Antibodies 35

Cell culture 35

Transfection of cells 38

Growth curve of cells 39

Electron microscopy 39

Immunofluorescence labeling 40

Scrape loading/Dye transfer 40

Immunoblotting 41

Northern blotting 42

Southern blotting 44

RNase protection assays 45

CHAPTER 4

MORPHOLOGY AND CHARACTERISTICS OF THE CELLS 47

9L Cells 47

C6 Cells 48

Growth rates of 9L and C6 cells 48

CHAPTER 5

COMPARISON OF GAP JUNCTIONAL CHARACTERISTICS OF 9L AND C6 GLIOMA CELLS 55

Immunofluorescence labeling of gap junctions 55

Gap junctional ultrastructure in 9L and C6 cells 55

Dye coupling between cells 66

Immunoblotting for connexin43 66

Northern blot analysis 73

CHAPTER 6

TRANSFECTION OF 9L GLIOMA CELLS AND INHIBITION OF CONNEXIN43 SYNTHESIS 81

Transfection of 9L cells 81

Loss of connexin43 gap junctions in transfected cells 86

Expression of sense and antisense connexin RNA 108

Gap junctional communication in the transfected cells after antisense RNA expression 115

Comparison of growth rates of transfected and untransfected cells 120

CHAPTER 7

DISCUSSION 125

Comparison of gap junctions in 9L and C6 rat glioma cells 125

Antisense expression for inhibition of gap junctions 127

Gap junctions and growth of rat glioma cells 132

CHAPTER 8

LITERATURE CITED 134

LIST OF TABLES

Table. 2.1.	Connexins from different organisms	12
Table. 2.2.	Expression of different connexins in different cells, tissues, and organs of different species	17
Table 2.3.	Factors affecting the connexin expression and gap junctional intercellular communication in different cells and tissues	26
Table. 4.1.	Dimensions of the 9L and C6 cell-bodies, processes, and their respective nuclei	49
Table. 6.1.	Effect of zinc treatment on the number and size of gap junctions between cells of 9L and AB5 cultures	99

LIST OF FIGURES

Figure. 2.1.	Topological map of connexin molecule	13
Figure. 3.1.	Diagram showing the relevant parts of plasmid T7/T3 291	36
Figure. 3.2.	Map of antisense connexin43 RNA synthesizing plasmid pNuTBGHE 914	37
Figure. 4.1.	Phase-contrast micrographs of 9L and C6 rat glioma cells	51
Figure. 4.2	Comparison of growth of 9L and C6 cells in culture	54
Figure. 5.1.	Immunofluorescence micrographs of 9L and C6 cells labeled with α_{1S} antibodies to connexin43	57
Figure. 5.2.	Electron micrographs showing gap junctions between 9L cells	59
Figure. 5.3.	Electron micrographs showing apposing membranes of C6 cells	62
Figure. 5.4.	Electron micrographs of lanthanum impregnated 9L and C6 gap junctions	65
Figure. 5.5.	Fluorescence photomicrographs of scrape loading/ dye transfer experiments in 9L and C6 cells	68
Figure. 5.6.	Immunoblotting of 9L and C6 cell alkali insoluble proteins for connexin43	70
Figure. 5.7.	Immunoblotting of whole cell homogenates and alkali insoluble proteins of 9L and C6 cells	72
Figure. 5.8.	Expression of Cx43 mRNA in 9L and C6 cells.	75
Figure. 5.9.	Relative expression of Cx43 mRNA in 9L and C6 cells.	77
Figure. 5.10.	Detection of Cx26 mRNA in mouse liver, 9L cells and C6 cells	79
Figure. 6.1.	G418 (Geneticin) sensitivity of 9L cells	83
Figure. 6.2.	Southern blot of untransfected 9L and transfected AB5 and another transfected AC4 genomic DNA	85
Figure. 6.3.	Immunofluorescence labeling of AB5 cells after antisense expression by zinc treatment	88
Figure. 6.4.	Confocal laser scanning microscopy images of immuno-fluorescence labeled AB5 cells after zinc treatment	91
Figure. 6.5.	Confocal laser fluorescence scanning images of immunolabelling of AB5 cells for connexin43 after zinc and cadmium treatment	94
Figure. 6.6.	Confocal laser fluorescence scanning images of immunolabelling of 9L cells for connexin43 after zinc treatment	96

Figure. 6.7.	Effect of antisense induction by zinc treatment on the number of gap junctions in 9L and AB5 cells	101
Figure. 6.8.	Effect of antisense induction by zinc treatment on the size of immunolabelled gap junction spots of 9L and AB5 cells	103
Figure. 6.9.	Immunoblotting of crude cell homogenates of AB5 cells after zinc treatment.	105
Figure. 6.10.	Immunoblotting of crude cell homogenates of 9L cells after zinc treatment	107
Figure. 6.11.	Northern blot analyses of 9L RNA with Cx43 and β-actin probes	110
Figure. 6.12.	Northern blot analyses of AB5 RNA with Cx43 and β-actin probes	112
Figure. 6.13.	RNase protection assay for antisense RNA detection in zinc treated AB5 cells	114
Figure. 6.14.	Scrape loading/ dye transfer of Lucifer Yellow CH fluorescent dye for intercellular dye transfer in AB5 cells	117
Figure. 6.15.	Scrape loading/dye transfer of Lucifer Yellow CH fluorescent dye for intercellular dye transfer in untransfected 9L cells	119
Figure. 6.16.	Comparison of growth of untreated and zinc-treated 9L and AB5 cells in culture	122
Figure. 6.17.	Multiple regression graph for growth rate comparison of untransfected and transfected cells after zinc treatment	124

LIST OF ABBREVIATIONS

ACTH	Adrenocorticotropic hormone
ATCC	American type culture collection
bFGF	Basic fibroblast growth factor
bp	Base pair
BSA	Bovine serum albumin
cAMP	Cyclic AMP
CDI	Cyclin-dependent kinase inhibitor
CDK	Cyclin-dependent kinase
cDNA	Complementary deoxyribonucleic acid
CMTX	Charcot-Marie-Tooth Disease (X-linked)
CNS	Central nervous system
Cx	Connexin
dCTP	Deoxycytidine triphoshphate
DDT	Dichloro diphenyl trichloroethane
DEPC	Diethyl pyrocarbonate
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
ECL	Enhanced chemiluminescence
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
EGTA	Ethylene glycol-bis-(b-aminoethylether)-N,N,N',N'-tetraacetic acid
FITC	Fluorescein isothiocyanate
FSH	Follicle stimulating hormone
GFAP	Glial fibrillary acidic protein
hCG	Human chorionic gonadotropin
HEPES	N-2-Hydroxyethylpiperazine-N'-2-ethanesulfonic acid
HPRT	Hypoxanthine phosphoribosyl transferase
IL-1	Interleukin-1
MEM	Minimal essential medium
MIP26	Membrane intrinsic protein 26
MOPS	4-Morpholinepropanesulfonic acid
mRNA	Messenger ribonucleic acid
NaOAc	Sodium acetate
NTP	Nucleotide tri-phosphate
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PDGF	Platelet-derived growth factor
PIPES	1,4-Piperazine bis(ethanesulfonic acid)
PMA	Phorbol 12-myristate 13-acetate
PMSF	Phenylmethylsulfonyl fluoride
PTH	Parathyroid hormone
RNA	Ribonucleic acid
RPA	Ribonucleic acid protection assay
SDS	Sodium dodecyl sulphate
SSC	Standard sodium citrate solution
TBS	Tris-buffered saline
TE	Tris-EDTA buffer
TEM	Transmission electron microscopy
TGFβ	Transforming growth factor beta

TPA	12-O-Tetradecanoylphorbol-13-acetate
TSH	Thyroid-stimulating hormone

Chapter 1

INTRODUCTION

Proliferation of cells is one of the many characteristics of neoplastic changes in tissues. Although aggressive proliferation of cells is an attribute of cancer cells, it is not alone sufficient to make cells cancerous (Hunter and Pines, 1994). Multiple genetic changes and cellular transformation are required for tumorigenesis (Hartwell and Kastan, 1994; Rabbitts, 1994). The genes involved in tumorigenesis include cell cycle control genes (Sager, 1992; Hartwell and Kastan, 1994; Hunter and Pines, 1994), growth factors, receptors of growth factors, molecules on the signal-transducing pathway of these receptors (Rozengurt, 1992; Baserga, 1994) and transcription factors (Rabbitts, 1994). Cell proliferation is directly related to the number of cells undergoing division in a cell population and the rate at which they are dividing. Cell division is under strict regulatory control at various 'check-points' of the highly organized cell cycle (Murray and Hunt, 1993). Mutations in these regulatory genes lead to genetic instability and increased probability of tumorigenesis.

Cell growth and tumorigenesis are also thought to be controlled by cell-to-cell contacts (Takeichi, 1991; Sporns *et al.*, 1995; reviewed by Loewenstein, 1987; Mesnil and Yamasaki, 1993; Hülsken *et al.*, 1994). The intercellular contacts are mediated by a number of cell adhesion molecules (see Malhotra, 1983; Stevenson and Paul, 1989; Sherman *et al.*, 1994; Hülsken *et al.*, 1994; Koch and Franke, 1994). However, direct cell-to-cell communication by low-resistance intercellular channels, termed gap junction channels, also exists between the cells in organized tissue. These gap junctions could affect the growth and proliferation of cells by passing regulatory molecules through them (Loewenstein, 1979; Loewenstein, 1987; Loewenstein and Rose, 1992). Gap junctions are intercellular channels that allow the non-selective passage of small molecules (less than 1 kD; reviewed by Loewenstein, 1979). Gap junctional channel formation occurs by alignment of two hemichannels from the apposing membranes of adjacent cells. The hemichannel or connexon is an assembly of six identical protein subunits, the connexin (Cx) subunits (Goodenough, 1976; Casper *et al.*, 1977; reviewed by Bennett *et al.*, 1991; Kumar, 1991; Kumar and Gilula, 1992; Stauffer and Unwin, 1992).

Gap junctions are ubiquitous in vertebrates and invertebrates with certain exceptions, such as mature skeletal muscle and spermatocytes. A number of different connexins have been identified to date which constitute a multigene family of related but distinct members (Willecke *et al.*, 1991 a; Kumar, 1991; Kumar and Gilula, 1992) and connexins are expressed in temporally and spatially regulated manner.

A great deal of effort has been expended to understand the structural components of gap junctions, their arrangement in channel formation, tissue distribution, and their regulation. These efforts have produced detailed information on the structure of the gap junction channels, electrical conduction properties, and different levels of regulatory controls ranging from the synthesis of the transcripts for connexin to the opening and closing of the channels. However, two distinct areas of research have shown meager results; these are (a) assembly of connexins into the hexameric connexon and ultimately in gap junction formation, and (b) specific role(s) of gap junctions in the cellular physiology of non-excitable cells. Progress in delineating the processes of assembly and intracellular transport has been dependent on the availability of molecular probes such as specific antibodies which have now become available (Hertzberg, 1985; Kumar and Gilula, 1992).

The role of gap junctions in electrotonic coupling of neurons and synchronization of excitable cells such as cardiac and smooth muscle cells has been well established (reviewed by Bennett, 1973; Sheridan and Atkinson, 1985). However, their role in the non-excitable cells has long been a subject of speculations based on circumstantial evidence except for a finding that mutations in a Schwann cell connexin gene may lead to Charcot-Marie Tooth disease of the peripheral nervous system (Bergoffen *et al.*, 1993 a; 1993 b) which leads to nerve degeneration. In another recent report, Reaume *et al.* (1995) targeted a mutation in a connexin gene (connexin43) of mouse and observed cardiac malformation during embryonic development.

Gap junctions also have been implicated in loss of growth control and tumor suppression (reviewed by Loewenstein, 1979; Bertram, 1990; Loewenstein and Rose, 1992). Correlative evidence for this hypothesis emanates from studies (see reviews by Loewenstein, 1979; Bennett *et al.*, 1991; Loewenstein and Rose, 1992) that showed that (a) many tumor cells are defective in gap junctional communication, (b) viral transformation of cultured cells results in the loss of gap junctions, and (c) tumor-promoter agents such as TPA inhibit gap junctional communication. It has been hypothesized (Loewenstein, 1979; Loewenstein and Rose, 1992) that growth regulatory

molecules can diffuse through the gap junction channels and exert their growth regulatory effects on neighboring cells. Regulatory molecules such as calcium ions, inositol phosphate, or cyclic nucleotides can, in theory, may pass from one cell to another through these channels, but the identity of these regulatory molecules has not been established. However, the idea of a direct role of gap junctional communication as a primary growth regulatory mechanism is controversial because of the following reasons; (a) many tumor cells and malignant cell lines do form gap junctions and exhibit intercellular communication (Bennett *et al.*, 1991), (b) during embryonic development gap junctions are formed as early as the eight-cell stage, and yet cell proliferation during embryogenesis is more rapid than in many tumors, (c) "knock-out" mice lacking a connexin43 gene (a gap junction gene which is expressed as early as the eight-cell stage of embryogenesis) developed to birth and showed only cardiac malformations (Reaume *et al.*, 1995). By experimentally causing the overexpression of connexin43 in the connexin-deficient C6 rat glioma cell line, it has been shown that the growth of cells decreased in cultures (Zhu *et al.*, 1991). Thus a direct relationship between gap junctional intercellular communication and the growth of C6 cells was proposed.

The ubiquitous presence of gap junctions and their relatively conserved molecular structures indicate their importance in vertebrate physiology, yet the tissue- and stage-specific expression of different connexins argues against them having a common role in different tissues. Moreover, the function of gap junctional communication may be supplementary to other cellular regulatory mechanisms because the loss of gap junctional potential does not affect cellular viability. That gap junctions are not indispensable has been shown by the development of "knock-out" mice to full-term (Reaume *et al.*, 1995); the mutation in connexin43 which is expressed even at eight-cell stage in the mouse embryo did not affect embryo development other than to produce cardiac malformations. It is known that different connexins impart distinct electrophysiological properties to the gap junctional channels in which they occur (Bennett *et al.*, 1991; Bennett and Verselis, 1992). Therefore, it is possible that a multiplicity of connexins provide supplementary functions in gap junction channels, with certain 'general' roles shared by all the channels and 'specific' roles restricted to different connexin isoforms. A major problem in assessing the effects of gap junctions has been the lack of a reliable method of inhibiting gap junction formation *in situ* in cells (Bennett *et al.*, 1991; Goodenough and Musil, 1993). A number of pharmacological agents cause closure of these channels, or downregulation of connexin synthesis, but they tend to be non-specific in their actions.

In the central nervous system (CNS) glia comprise the major population of cells. Gap junction formation and cell-to-cell coupling is known to exist in these cells (reviewed by Bennett *et al.*, 1991; Dermietzel and Spray, 1993). Metabolic cooperation and sharing of organic molecules and inorganic ions has been suggested to be the major role of the gap junctions in astrocytes (Bennett, 1973; Sheriden and Atkinson, 1985; Dermietzel and Spray, 1993). A salient feature of gap junctional function in brain physiology is the buffering of potassium ions during neuronal activity (Orkand *et al.*,1966; Karwoski *et al.*, 1989). Astrocytes are believed to provide a sink for potassium ion efflux into the extracellular milieu (see reviews by Newman *et al.*, 1984; Dermietzel and Spray, 1993) inasmuch as they offset the local increase in potassium ions through the uptake and spatial redistribution of potassium ions from the extracellular space. Gap junctions may be important in reactive astrocytes during the process of astrogliosis possibly by facilitating cell-to-cell communication (Alonso and Privat, 1993). A role for gap junctions in the growth and proliferation of glial cells and their tumors also has been proposed (Zhu *et al.*, 1991; Naus *et al.*, 1992; Zhu *et al.*, 1992). A direct method of investigating the latter possible role of gap junctions would be to use "knock-out" mutations and antisense techniques. Transfection of cells to express antisense RNA could inhibit connexin expression in a direct and specific fashion, so as to reveal the regulatory role of gap junctions in controlling cell growth and proliferation.

Objectives:

In the present study, the main objective was to test the hypothesis that gap junctions regulates the growth of glioma cells *in vitro*. To this end, I have compared 9L and C6 rat glioma cell lines for their levels of gap junctional communication, relative to their growth rates in culture. Also, the 9L rat glioma cells were transfected with an inducible antisense RNA expressing vector to assess the effect of gap junction inhibition on cell growth. The following strategies have been employed in these studies;

(1) Characterization of gap junctional components in 9L and C6 rat glioma cell lines using immunological methods such as immunofluorescent labeling of culture-grown cells and immunoblotting of cellular proteins, and RNA analyses by Northern blot analyses. Assessment of gap junctional intercellular communication by dye transfer assays, and by transmission electron microscopy.

(2) Comparison of growth rates of 9L and C6 glioma cells in relation to their gap junctional characteristics by plotting growth curves.

(3) Transfection of 9L glioma cells with antisense connexin43 plasmid to experimentally inhibit gap junction formation by inhibiting connexin43 expression.

(4) Characterization of transfected glioma cells for inhibition of gap junctions using immunological, molecular, and dye-transfer techniques and to assess the effect of gap junction inhibition on the growth rate in transfected cells.

The results obtained from this study clearly show that 9L and C6 rat glioma cells display disparate constitutive capacities for connexin synthesis and gap junctional communication. However, their growth rates in cultures are very similar. Induction of antisense RNA expression in 9L cells resulted in a loss of gap junctions which was accompanied by a loss of intercellular dye-transfer. The growth rate of transfected cells was reduced as compared to non-induced cells. Therefore, gap junctional communication may play a role in growth regulation but may not be the major determining factor in the rate of growth of these cells *in vitro*.

Chapter 2

REVIEW OF LITERATURE

Cells in organized tissue make various types of cell-to-cell contacts such as desmosomes, adherens junctions, tight junctions and gap junctions (Farquhar and Palade, 1963; reviewed by Malhotra, 1983; Stevenson and Paul, 1989; Sherman *et al.*, 1994; Hülsken *et al.*, 1994; Koch and Franke, 1994). Tight junctions and desmosomes provide apical-basal partitioning in epithelial cells and anchoring functions, respectively, whereas gap junctions are contacts which allow participating cells to communicate and exchange materials directly through intercellular channels. The latter are the subject of the present study.

In this chapter, literature pertaining to historical perspective of discovery of gap junctions and their molecular constituent proteins, and structure of gap junctional channels is reviewed. A summary of the current knowledge of the roles played by gap junctions in cellular physiology is also presented.

Gap junctions and their historical perspective

Direct intercellular communication has long been studied by electrophysiologists, cell physiologists, and embryologists because such communication plays important roles in passage of molecular signals between cells, maintenance of metabolic homeostasis in tissues, and formation of gradients of molecules during embryogenesis. The existence of gap junctions was first indicated by a study of crayfish neurons (Furshpan and Potter, 1957). Apart from the chemical transmissions at the synapses, unidirectional transmission of electrotonic pulses between pre- and post-synaptic neurons was described at the membrane junctions of some neurons. These 'electrical synapses' were ultrastructurally different from the chemical synapses (Bennett *et al.*, 1963). With the development of electron microscopic techniques, morphological correlates for such communicating junctions were discovered which, in turn, led to further physiological insights. Dewey and Barr (1962; 1964) described nexuses (discrete regions of intercellular contacts of variable size where the plasma membranes of adjacent cells 'fused' and excluded extracellular fluid) in dog intestine smooth muscles and in other animal tissues. Although Dewey and Barr (1962) discerned a correlation between nexuses and the

passage of intercellular current in smooth muscle cells, Bennett *et al.* (1963) showed that electrotonic transmission between spinal neurons in the electric fish was correlated with a distinctive apposition of cell processes involving "membrane fusions". A historical background of the advances in the field of gap junction research has been published by Robertson (1981).

Karrer (1960), Dewey and Barr (1962; 1964), Robertson (1963) and Farquhar and Palade (1963) described intercellular junctions in different tissues by transmission electron microscopy (TEM) that were later recognized as gap junctions. Farquhar and Palade (1963) also proposed different terms for various intercellular junctions but they could not differentiate between 'gap junctions' and 'tight' junctions because of the limited resolution of the technique used in their studies. In the Maüthner cell synapses of goldfish, Robertson (1963) described hexagonal assemblies in the plasma membranes associated with intercellular junctions. Although he considered them to be possible "aqueous channels across the unit membrane", he could not draw any conclusion about these structures. Using a different technique of negative staining with phosophotungstic acid of isolated rat liver membranes, Benedetti and Emmelot (1965) also reported a similar hexagonal array of subunits in the membranes. Revel and Karnovsky (1967) using colloidal lanthanum as an extracellular tracer showed that some intercellular junctions display minute gaps between external leaflets of plasma membrane which allow the penetration of colloidal lanthanum and have hexagonal arrangements in the membranes, best displayed in grazing sections of the membranes. Tight junctions, however, completely obliterate the extracellular space and lack both lanthanum permeability and demonstrable hexagons. To differentiate them from the tight junctions, they coined the term gap junctions. Revel *et al.* (1967) later showed that "nexus" seen by Dewey and Barr (1962) also shared the same characteristics. The term "gap junction" is now almost universally used despite suggestion of other names such as nexus (Dewey and Barr, 1962) and macula communicans (communicating junctions, Simionescu *et al.*, 1975).

By TEM of stained thin sections, gap junctions appear as regions of close apposition of the plasma membranes of adjacent cells. The profile of gap junctions in thin section electron micrographs can be variable, the variation arising from the method of fixation and sample preparation (see review by Larsen, 1977). A typical gap junction profile is described as septalaminar, resulting presumably from the close apposition of two adjacent unit membranes. The entire width of the junction is usually 15-18 nm, and the space between the membranes is approximately 2-4 nm. A plasma membrane in

stained thin sections is seen as three layers (two dark outer hydrophilic domains sandwiching an electron-transparent lighter layer). Therefore, the seven-layered gap junction profile arises from six layers of the adjacent plasma membranes and a layer between these membranes. Although septalaminar structure is thought to confirm the gap junctional profile, many earlier reports described pentalaminar profiles (Revel and Kornovsky, 1967; reviewed by McNutt and Weinstein, 1973; Larsen, 1977). Recent reports, also have shown pentalaminar structures by conventional electron microscopy (Naus *et al.* 1993).

Later, freeze fracture technique clearly demonstrated the presence and distribution of gap junctions in the cell membrane. Using freeze-fracture technique, Kreutziger (1968) first demonstrated gap junctions as a lattice of hexagonal intramembrane particles. This technique thus provided the definitive morphological evidence for gap junctions in tissues. In freeze-fractured replicas, the gap junctions appear to be lattices of hexagonal intramembrane particles on the P-face (protoplasmic face), whereas the E-face (ectoplasmic face) displays corresponding pits. The center-to-center spacing between these hexagonal particles is ~8 to 9 nm. These freeze-fracture studies of gap junctions led to the concept that integral plasma membrane proteins in the two apposing plasma membranes join together to form the communicating channels.

These gap junction proteins were given the term connexins by Goodenough (1974) and the hexagonal components of gap junctions were later called connexons (Goodenough, 1976). McNutt and Weinstein (1970) correlated the results from thin-section- and freeze-fracture-electron microscopy to provide a model of intercellular channels crossing plasma membranes of both the participating cells. Subsequently, structural studies on the isolated gap junctions using high resolution electron microscopy, electron diffraction and X-ray diffraction revealed that connexons are cylindrical hexameric assemblies of connexin molecules delineating an axial aqueous channel (Makowski *et al.*, 1977; Casper *et al.*, 1977; Unwin and Zampighi, 1980) and each connexon is connected across the gap to a second hexamer forming a single cell-to-cell channel.

Molecular constituents of gap junctions

The identity of gap junction proteins (connexins), their numbers, and molecular weights have been subject to various interpretations and were debated in the past. Connexins of different molecular weights were reported from the same tissue by different

researchers (see Bennett and Goodenough, 1978; Robertson, 1981; Willecke and Traub, 1990). The controversy arose because the migration of connexins on SDS-PAGE were anomalous (Kumar and Gilula, 1986; Green *et al.*, 1988). The presence of relatively constant morphological features of gap junctions in spite of several different connexins in the same or different tissues, and enumeration of aggregates and proteolytic fragments of these connexins as genuine proteins augmented the confusion.

Identification of gap junction proteins: the connexins

Most of the studies on mammalian gap junctions have been carried out on liver, heart, and lens tissue because gap junctions are abundant in these tissues. Gap junction plaques have been isolated using non-ionic detergent extraction methods from mouse and rat livers (Evans and Gurd, 1972; Goodenough and Stockenius, 1972; Hertzberg and Gilula, 1979; Sikerwar and Malhotra, 1983). A better yield of gap junction plaques and proteins has been reported by Hertzberg (1984) using alkali extraction of the plasma membrane. Improvement in the method of gap junction protein purification was crucial in obtaining consistent, reproducible data on the SDS-PAGE, and large quantities of protein for amino acid sequencing and the generation of antibodies.

Electrophoretic analyses of connexins from purified gap junctions revealed the molecular sizes of these constituents. Purification of mouse and rat liver gap junctions indicated two major polypeptides, with a major protein of Mr 26 to 28 kD, and a lesser amount of a 21 kD protein (Henderson *et al.*, 1979; Finbow *et al.*, 1980). This major liver gap junction protein is now known as connexin32 (Cx32, 32 kD) based on its cDNA analysis (Beyer *et al.*, 1987). The Cx32 migrates anomalously on the SDS-PAGE of differing polyacrylamide concentrations (Green *et al.*, 1988).

The Mr 21 kD protein was initially thought to be a proteolytic product of Cx32 because both proteins had a common tryptic peptide (Henderson *et al.*, 1979). Traub *et al.* (1982) showed that in liver, antibodies to Cx32 and to the 21 kD protein did not cross-react mutually, thus indicating two different proteins in liver gap junctions. Partial amino acid sequencing of these proteins (Nicholson *et al.*, 1987), and later cDNA isolation (Zhang and Nicholson, 1989) showed that these proteins were indeed different. The 21 kD protein is now regarded as a gap junction protein with a deduced molecular weight of 26 kD and termed connexin 26 (Cx26).

In heart tissue, initially a number of gap junction protein bands were described (Kensler and Goodenough, 1980; Manjunath *et al.*, 1982). In another analysis of rat myocardial gap junctions (Gros *et al.*, 1983) the major gap junction protein was found to be a 28 kD polypeptide. This polypeptide was believed to have originated from a 30 kD protein after proteolysis. However, further careful analyses of the proteins in the presence of protease inhibitors revealed that the rat heart gap junctions contain a 43 kD protein (Cx43; Manjunath and Page, 1984). This has been confirmed by deduced molecular weight from isolated cDNA (Beyer *et al.*, 1987).

In vertebrate lens tissue two proteins, major intrinsic protein 26 (MIP26) and MP70 were thought to be the representative gap junction proteins (discussed by Revel *et al.*, 1985). Amino acid sequence analysis (Nicholson *et al.*, 1983), and molecular cloning and cDNA analysis (Gorin *et al.*, 1984) have shown that MIP26 is not related to the connexins. MP70 on the other hand was found to be a member of connexin family first by sequence homology of the partial amino acid sequences (Kistler *et al.*, 1988) and later by cloning and cDNA analysis. It has been correlated to mouse Cx50 on the basis of extensive sequence homology (White *et al.*, 1992). Another member of the connexin family, Cx46 was isolated from a rat cDNA library (Paul *et al.*, 1991). Recently, a chick eye lens connexin (Cx 45.6, 45.6 kD) has been identified (Jiang *et al.*, 1994), which is specifically expressed in the lens fibers.

Connexins from other vertebrates, notably chick and *Xenopus* (Gimlich *et al.*, 1988; Ebihara *et al.*, 1989) have also been identified. These connexins show a high level of sequence homology to mammalian connexins (Beyer *et al.*, 1990).

cDNA analysis of connexins and the connexin multigene family

The first cDNAs for gap junctions were isolated independently by Paul (1986) and Kumar and Gilula (1986). In one approach (Paul, 1986) antibodies to Cx32 were used to screen cDNA expression library from rat liver cDNA, whereas in the other approach (Kumar and Gilula, 1986) oligonucleotide probes (based on the partial amino acid sequence of liver connexin) were used to screen a human liver cDNA library. Analysis of these cDNA sequences suggested that both rat and human liver connexins encoded a 32 kD protein. Subsequently, a cDNA for a 43 kD protein (Cx43) was isolated from a rat myocardial library using a low stringency hybridization technique. Use of synthetic oligonucleotides (based on known amino acid sequences as probes) and low

stringency screening has resulted in the isolation of a number of other connexins (Table 2.1).

A topological model based on the deduced amino acid sequence and theoretical hydropathy plot of the Cx32 protein has been constructed. The model predicted four transmembrane regions in the Cx32 molecules, which has been supported by the analysis of the protected peptides after protease treatment of isolated gap junction plaques. The hydropathy plot of the Cx43 amino acid sequence also yielded the same hypothetical topological model as derived for Cx32 (Beyer *et al.*, 1987). Experimental support for this model has come from the immunoreactivity of antibodies raised to specific synthetic peptide regions of Cx32 (Milks *et al.*, 1988), Cx43 (Yeager and Gilula, 1992; Zhang and Nicholson, 1994), and Cx26 (Zhang and Nicholson, 1994).

Topological model of connexin molecules

Topological models for several connexins have been suggested by Kumar and Gilula (1992). The general arrangement of connexin molecules show the following features (Fig. 2.1; Beyer *et al.*, 1990; Willecke and Traub, 1990; Bennett *et al.*, 1991; Kumar, 1991; Kumar and Gilula, 1992; Stauffer and Unwin, 1992);

(1) both the amino- and the carboxy-terminal ends of the protein face the cytoplasm,

(2) the polypeptide traverses the lipid bilayer of the plasma membrane four times,

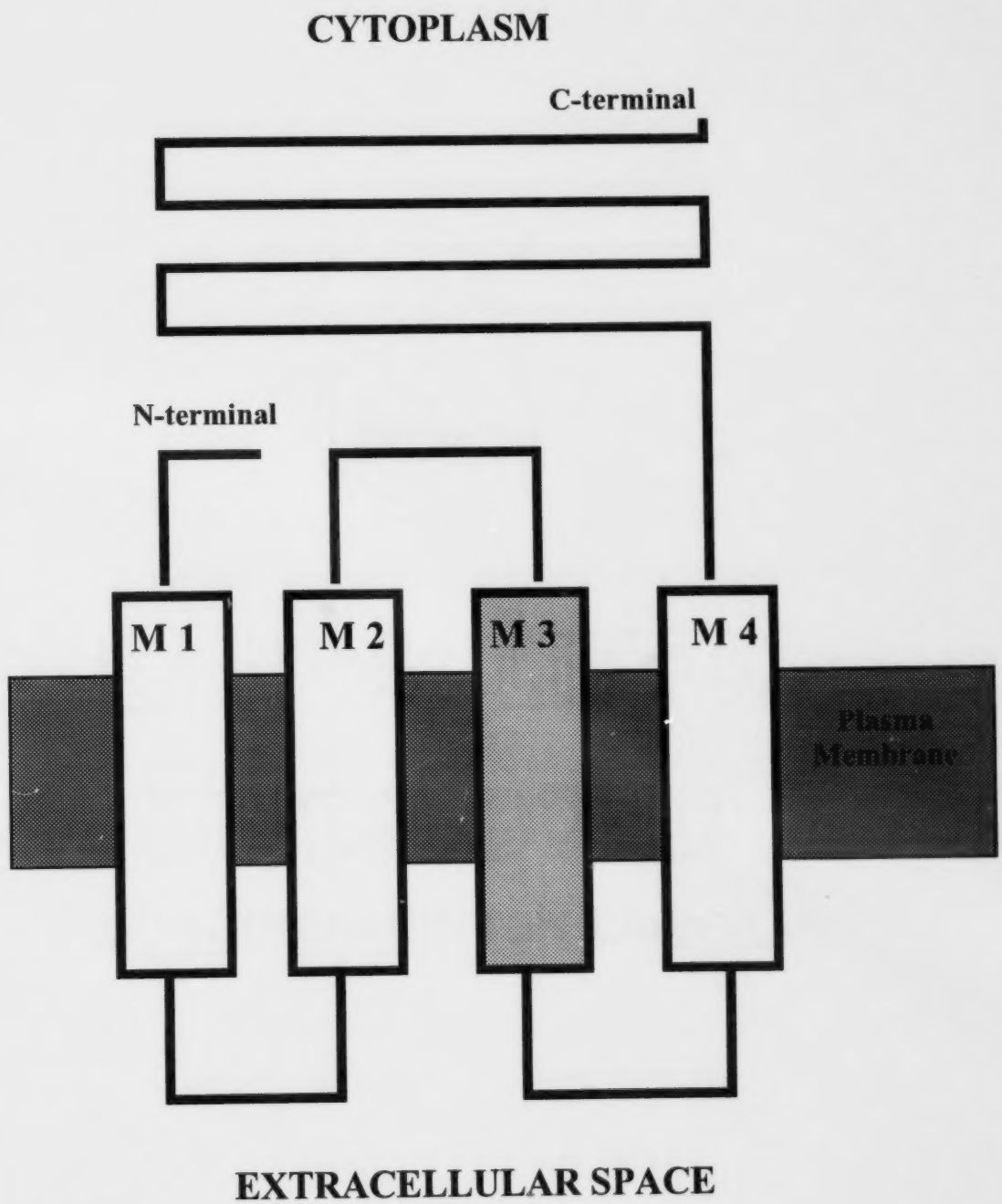
(3) the third transmembrane domain (M3) has an amphipathic character that would be expected from a channel-lining domain which allows the passage of hydrophilic molecules,

(4) a set of three cystein residues with a characteristic spacing is present in the two putative extracellular loops. Although intercellular disulfide linkages are not found, intramolecular bonds may be formed (Dupont *et al.*, 1989; Rahman and Evans, 1991; John and Revel, 1991)

Table. 2.1. The connexins cloned from different animals using various strategies. This table shows the names of the connexins and their reported sizes of mRNA.

Organism	Connexin	Size of mRNA	Reference
Human	Cx32	1.6 kb	Kumar and Gilula, 1986
	Cx43		Fishman et al., 1991
	Cx37		Reed et al., 1993
Rat	Cx32	1.6 kb	Paul, 1986
	Cx43	3.2 kb	Beyer et al., 1987
	Cx26	2.5 kb	Zhang and Nicholson,1989
	Cx46	2.8 kb	Paul et al., 1991
	Cx31	1.7 kb	Hoh et al., 1991
	Cx31.1	1.5 kb	Haefliger et al., 1992
	Cx33	2.3 kb	Haefliger et al., 1992
	Cx37	1.5 kb	Haefliger et al., 1992
	Cx40	3.4 kb	Haefliger et al., 1992
	Cx40		Beyer et al., 1992
	Cx30.3		Tucker and Barajas, 1994
Mouse	Cx37	1.7 kb	Willecke et al., 1991 b
	Cx32		Willecke et al., 1991 a
	Cx43		Willecke et al., 1991 a
	Cx31	1.9 kb	Henneman et al., 1992 b
	Cx45	2.3 kb	Henneman et al., 1992 b
	Cx30	1.9 kb/3.2 kb	Henneman et al., 1992 b
	Cx31.1	1.6 kb	Henneman et al., 1992 b
	Cx40	3.5 kb	Henneman et al., 1992 a
	Cx50 (MP70)	8.5 kb	White et al., 1992
Dog	Cx40	2.6 kb	Kanter et al., 1992
	Cx45	2.1 kb	Kanter et al., 1992
Bovine	Cx44	2.5 kb	Gupta et al., 1994
Chicken	Cx43	3.0 kb	Musil et al., 1990
	Cx41.7	3.0 kb	Beyer et al., 1990
	Cx45.4	2.0 kb	
	Cx56		Rup et al., 1993
	Cx45.6	6.4 kb/9.4 kb	Jiang et al., 1994
Xenopus	Cx30	1.5 kb	Gimlich et al., 1988
	Cx43	2.6 kb	Ebihara et al., 1989
	Cx38	1.5 kb	Gimlich et al., 1990

Fig. 2.1. Topological map of connexin molecule based on the amino acid sequence analysis (Not drawn to scale).



Although the secondary structure of connexins is not known, based on the primary structure of connexin both α-helical and β-pleated structures can be predicted (Kumar and Gilula, 1992). On the basis of the results from circular dichroism the primary structure of connexins is thought to be α-helical (Kumar and Gilula, 1992). Among all the connexins, the amino-terminal region, the putative transmembrane regions, and extracellular domains are highly conserved. The carboxy-terminal region and the intracellular loop connecting the second and third transmembrane segments are the most divergent regions among connexins (Stevenson and Paul, 1989; Beyer *et al.*, 1990; Bennett *et al.*, 1991; Sáez *et al.*, 1993). The length of the carboxy-terminal end varies greatly among different connexins (18 amino acids in Cx26, 256 amino acids in Cx46), whereas the cytoplasmic loop shows slight variation in its length. These features suggest that whereas similarities among connexins allow them to form heterotypic channels, their differences imply distinctiveness and specificity to the functions of these channels.

Although heterotypic channel formation is possible, all the combinations are not allowed. In a *Xenopus* oocyte expression system, Cx40 could not pair with Cx43 whereas each did pair up to form gap junction channels with Cx37 (Bruzzone *et al.*, 1993). In another study chimeric connexins were experimentally synthesized by exchanging different molecular domains of rat Cx43 and *Xenopus* Cx38. When these chimeric connexins were expressed in *Xenopus* oocytes (Bruzzone *et al.*, 1994) and allowed to make gap junctions in homotypic and heterotypic combinations, it was found that most of these connexins were devoid of functional activity as judged by electrical coupling. This study indicated that connexins are not composed of functionally exchangeable regions and that multiple domains influence the interactions between connexins in adjacent cells. This provides a regulatory mechanism for cell-to-cell communication in different tissues.

Nomenclature of gap junction proteins

Gap junction proteins were originally named according to their tissue of origin and apparent molecular weights on SDS-PAGE (Bennett *et al.*, 1991) such as Cx32 was called the liver-type connexin and Cx43 was named the heart-type connexin. Because a connexin can be expressed in more than one tissue, nomenclature schemes have been modified. Based on the homology of different connexin cDNAs and their corresponding proteins it has been suggested that these related genes belong to a new gene family; each

member of this family is termed a connexin and the molecular weight, as deduced from cDNA analysis is used to distinguish the different members (Beyer *et al.*, 1987). Under this nomenclature system, the calculated molecular weight is chosen for specification of each individual connexin because the electrophoretic mobilities of these proteins vary depending on the experimental conditions (Green *et al.*, 1988). It is pertinent to mention that all known connexin genes belong to vertebrates; no connexin gene has been reported from an invertebrate thus far.

A Greek nomenclature scheme for the connexin gene family, based on genetic homology and structural relationships among the members also has been proposed. According to this system, connexins are divided into two classes: α and β, which are based on the presence of a specific amino acid motif in the polypeptide region that would putatively form the channel lining (Milks *et al.*, 1988). This scheme has been modified to accomodate members that show overall homology to another class despite the presence of the motif for a given class (Kumar and Gilula, 1992). The criteria to include a gene in the connexin multigene family are outlined by Kumar and Gilula (1992). Although this scheme is cogent and useful, the conventional nomenclature of using Cx prefix followed by the deduced molecular weight is popular and more commonly applied. However, the Human Gene Nomenclature Committee has now adopted the α and β distinction (Kumar and Gilula, 1992).

It is apparent from the Table 2.1 that connexins from different organisms have been given the same name despite their lack of 100% sequence homology. Therefore, a confusion about the identity of the connexins exists in the literature and the name of a connexin does not provide enough information without the knowledge of the organism it belongs to. The Greek nomenclature of Gilula and co-workers is, therefore, a suitable method because it identifies each connexin uniquely.

Connexin Genes and their Chromosomal Location

Connexin genes show one intron in the 5' non-coding region, whereas the coding region is uninterrupted (Sullivan *et al.*, 1993; Miller *et al.*, 1988). Chromosomal loci for several human and mouse connexin genes have been mapped (Willecke *et al.*, 1991 a; Fishman *et al.*, 1991; Hsieh *et al.*, 1991). These studies show that five human connexin genes and a pseudogene are situated on five different human chromosomes (Willecke *et al.*, 1991 a). Mouse connexin genes are similarly dispersed on different chromosomes (Hsieh

et al., 1991). Thus, the members of the connexin gene family are not organized in large clusters but are dispersed throughout the chromosomes.

Tissue distribution of gap junctions

Gap junctions in vertebrate tissues are almost universal in organized tissues with the exception of blood cells, mature skeletal muscle cells, and spermatocytes. Connexins have complex expression patterns; they are expressed in a tissue-, developmental- stage- and physiological-stage-specific manner. A given tissue may express more than one connexin and a given connexin can be expressed in more than one tissue. With the data obtained from comparative peptide mapping of connexins from different tissues it was suggested that different tissues might be comprised of distinct connexins, specific to each germ layer (Gros *et al.*, 1983). Generation of antibodies to connexins (Traub *et al.*, 1982; Janssen-Timmen *et al.*, 1983; Hertzberg, 1984; Warner *et al.*, 1984) helped in mapping the distribution of the connexins in different tissues. Using immunofluorescence labeling, antibodies were shown to bind to punctate regions of the plasma membrane of hepatocytes (Hertzberg and Skibben., 1984; Janssen-Timmen *et al.*, 1983).

Availability of cDNA probes resulted in rapid screening of different tissue from many species. A list of the tissues expressing different connexins is compiled in the Table 2.2. This Table is not exhaustive, but lists a number of vertebrate tissues for their connexin expression. It is clear from the Table that more than one connexin can be expressed in a given tissue, and that the same connexin may be expressed by several tissues. It is also evident that in spite of the cell-specific expression of connexins, there is considerable overlap in their distribution, and the original belief that connexins are expressed in a germ layer-specific manner is no longer valid. It is notable, however, that two connexins, Cx33 and Cx50, show restricted expressions respectively in the testes and in eye lens. Of the connexins characterized to date, Cx26 is unique in that it is usually expressed in conjunction with other members of the family (Zhang and Nicholson, 1994). When several types of connexins are expressed in the same cell, they may colocalize in the same gap junctional plaque, but the presence of different connexins in the same connexon is not known (Zhang and Nicholson, 1994).

It should be noted that many of the studies on the tissue distribution of connexins have used the detection of connexin mRNA as a criterion for connexin expression. Although there is a good correlation between the levels of connexin mRNA and the

Table. 2.2. Expression of different connexins in various cells, tissues, and organs of different species.

Tissue/Cell	Species	Connexin	References
Pigmented ciliary epithelial cells	Human	Cx43	Coca-Prados et al., 1992
	Bovine	Cx 43	
Osteoblast -like cells	Rats	Cx43	Schirrmacher et al., 1992
	Gunea pigs		
MDCK cells	Dog	Cx43	Berthoud et al., 1992
Uterine smooth muscle cells	Human	Cx43	Sakai et al., 1992
Mammary tumor cell line	Human	Cx26	Lee et al., 1992
Microvascular endothelial cells	Bovine	Cx43	Pepper and Meda, 1992
Atrial endothelium	Rat	Cx40	Bruzzone et al., 1993
Skin	Rat	Cx37, Cx31.1	Haefliger et al., 1992
Dermis and epidermis	Human	Cx43	Guo et al., 1992
Epidermis	Rat	Cx43, Cx26	Risek et al., 1992
	Rat	Cx43, Cx37, Cx26, Cx31.1	Goliger and Paul, 1994
	Rat	Cx43, Cx26	Risek et al., 1994
Hair follicles	Rat	Cx43, Cx26	Risek et al., 1992
Keratinocytes	Mouse	Cx43, Cx26	Brissette et al., 1991
Sebocytes	Rat	Cx43, Cx31	Risek et al., 1992
SNB motoneurons	Rat	Cx32	Matsumoto et al., 1991 Matsumoto et al., 1988
Brain	Rat	Cx43, Cx32	Micevych and Abelson, 1991
	Rat	Cx43	Vukelic et al., 1991
	Rat	Cx43	Dupont et al., 1991
	Mouse	Cx37	Willecke et al., 1991
	Mouse	Cx31	Hennemann et al., 1992 a
	Mouse	Cx45	Hennemann et al., 1992 a
	Rat	Cx43, Cx32, Cx26	Giaume et al., 1991
Sciatic nerve	Rat	Cx32	Bergoffen et al., 1993
Glial cells	Rat	Cx43	Yamamoto et al., 1990
Neurons	Rat	Cx32	Dermietzel et al., 1989
Astrocytes	Rat	Cx43	Dermietzel et al., 1989
	Mouse	Cx43	Giaume et al., 1991
	Rat	Cx43	Dermietzel et al., 1991
	Rat	Cx43	Naus et al., 1991

Tissue/Cell	Species	Connexin	References
Oligodendrocytes	Rat	Cx32	Dermietzel et al., 1989
			Micevych and Abelson, 1991
Pinealocytes	Rat	Cx26	Dermietzel et al., 1989
	Rat	Cx43, Cx26	Saez et al., 1991
Leptomeninges	Rat	Cx43, Cx26	Dermietzel et al., 1989
	Rat	Cx43, Cx26	Spray et al., 1991
Ependyma	Rat	Cx43, Cx26	Dermietzel et al., 1989
Olfactory	Mouse	Cx43, Cx32, Cx26	Miragall et al., 1992
Liver	Mouse	Cx32, Cx26	Nicholson et al., 1987
	Rat	Cx32	Kumar and Gilula, 1986
	Rat	Cx32	Paul, 1986
	Mouse	Cx26	Willecke et al., 1991
	Rat	Cx32, Cx26	Zhang and Nicholson, 1989
	Mouse	Cx32,Cx26	Zhang and Nicholson, 1989
NRK cells		Cx43	Musil and Goodenough, 1991
Lens	Rat	Cx46	Paul et al., 1991
	Bovine	Cx46	
	Bovine	Cx46	Tenbroek et al., 1992
	Bovine	Cx43	Reynhout et al., 1992
	Chick	Cx43	Musil et al., 1990
	Mouse	Cx50 (MP70)	White et al., 1992
	Bovine	Cx44	Gupta et al., 1994
	Chick	Cx45.6	Jiang et al., 1994
Kidney	Rat	Cx46	Paul et al., 1991
	Mouse	Cx37	Willecke et al., 1991
	Rat	Cx40, Cx37	Haefliger et al., 1992
	Rat	Cx32, Cx26	Zhang and Nicholson, 1989
	Rat	Cx30.3, Cx31	Tucker and Barajas, 1994
Heart	Rat	Cx43	Beyer et al., 1989
	Rat	Cx46	Paul et al., 1991
	Mouse	Cx37	Willecke et al., 1991
	Mouse	Cx45	Hennemann et al., 1992 b
	Rat	Cx40, Cx37	Haefliger et al., 1992
	Rat	Cx40, Cx43	Gourdie et al., 1993
Lung	Mouse	Cx37	Willecke et al., 1991
	Mouse	Cx45	Hennemann et al., 1992 a
	Rat	Cx40, Cx37	Haefliger et al., 1992
	Rat	Cx32, Cx26	Zhang and Nicholson, 1989
Spleen	Mouse	Cx37	Willecke et al., 1991

Tissue/Cell	Species	Connexin	References
	Rat	Cx32	Bergoffen et al., 1993
Intestine	Mouse	Cx31	Hennemann et al., 1992 b
	Rat	Cx32,Cx26	Zhang and Nicholson, 1989
Stomach	Rat	Cx32, Cx26	Zhang and Nicholson, 1989
Ovary	Rat	Cx40, Cx37	Haefliger et al., 1992
	Rat	Cx43	Risek et al., 1990
Testis	Rat	Cx37, Cx33	Haefliger et al., 1992
	Rat	Cx43	Risley et al., 1992
	Mouse	Cx43	
Seminiferous epithelia	Rat	Cx32, Cx26	Risley et al., 1992
Leydig cells	Mouse	Cx43	Varanda and de Carvalho, 1994
Uterine myometrium	Rat	Cx43	Risek et al., 1990
			Tabb et al., 1992
Endometrium	Rat	Cx32, Cx43	Risek et al., 1990
Bone cells	Rat	Cx43	Jones et al., 1993

respective protein products, the presence of mRNA does not necessarily indicate the expression of the functional protein (Kumar, 1991). Formation of functional gap junctions is dependent on correct phosphorylation of connexin molecules in cells (Musil *et al.*, 1991), and modulation in protein kinase activities may cause changes in the phosphorylation of connexin molecules resulting in closure of gap junctions (Musil and Goodenough, 1990).

Switching of connexin expression in a given cell type is seen during differentiation of thyroid epithelial cells (Munari-Silem *et al.*, 1994), mouse primary keratinocytes (Brissette *et al.*, 1994), differentiation of rat oval cells into hepatocytes (Zhang and Thorgeirsson, 1994), and during the development of rat fetal epidermis cells (Risek *et al.*, 1994). Connexin distribution is also temporally regulated in *Xenopus*; Cx43 and Cx38 mRNA are abundant in oocytes prior to ovulation and meiotic maturation, but Cx43 disappears upon maturation of oocytes. Cx38 persists until the early gastrula stage (Ebihara *et al.*, 1989; Gimlich *et al.*, 1990) then is replaced by Cx30. In mouse, the assembly of gap junctions starts during compaction in the eight cell stage; however the Cx43 transcripts are detected at the four-cell stage (Lo and Gilula, 1979; Nishi *et al.*, 1991; Gilula *et al.*, 1992).

Although the biological significance of these patterns of distribution is not understood, they are thought to provide physiological differences and regulatory control. It is becoming increasingly clear that functional properties of a channel are dependent on the intrinsic properties of its connexin as well as on those of the corresponding connexin from the other participating cell. This suggests that it is advantageous for the cells to regulate the expression of connexin genes in step with distinct differentiation or developmental programs (Willecke *et al.*, 1991 a). More recently, the unidirectional passage of fluorescent dyes in rabbit retina astrocytes to oligodendrocytes and Müller cells has been reported (Robinson *et al.*, 1993). The dye could not pass from either oligodendrocytes or Müller cells to the astrocytes, suggesting a potential for unidirectional signaling.

Gap junctions in the Central Nervous System (CNS)

Gap junctions were originally discovered in crayfish nerve cords and in goldfish Maüthner cells in structural and elecrophysiological studies (Furshpan and Potter, 1957; Bennett *et al.*, 1963; Robertson, 1963). Concurrently, the presence of gap junctions by

TEM was shown between neurons (Sotelo and Korn, 1978; Jaslov and Brink, 1987). Following the development of antibodies to connexin and cDNA probes, rapid progress was made in mapping the distribution of different connexins in the CNS (see review by Dermietzel and Spray, 1993). In the CNS, glial cells (especially astrocytes) constitute the bulk of the tissue. The astrocytes have been reported to be electrophysiologically coupled through junctional channels (Connors *et al.*, 1984). Immunohistochemical techniques (Dermietzel *et al.*, 1989; Yamamoto *et al.*, 1990) and *in situ* hybridization (Micevych and Abelson, 1991; Matsumoto *et al.*, 1991), have demonstrated that connexins are widespread in the brain and spinal cord. Connexins in the CNS are expressed in a cell-specific manner. Most of the expression is seen in astrocytes which express Cx43 whereas the oligodendrocytes express Cx32. Cultured primary astrocytes express Cx43 mRNA and protein, but not any other connexin (Giaume *et al.*, 1991; Dermietzel *et al.*, 1991). Cells from leptomeninges (Spray *et al.*, 1991) and ependyma (Dermietzel *et al.*, 1989) also express Cx43, both *in vivo* and *in vitro*. It has been reported that the regional distributions of Cx43 and Cx32 in different brain regions vary (Naus *et al.*, 1990; Lee *et al.*, 1994). Even the relative quantities of Cx43 in various brain regions have been found to differ which might be important in regulation of functions these cells perform (Nagy *et al.*, 1992). Additionally, it appears that cultured astrocytes from different brain regions show heterogeneity with regards to Cx43 expression and gap junctional communication (Batter, et al, 1992), and that cortical type 2 astrocytes do not express Cx43 or show junctional communication (Belliveau and Naus, 1994). Rat pinealocytes express Cx26, but not Cx43 or Cx32 (Sáez *et al.*, 1991), whereas the olfactory system, Cx43 is the major connexin expressed (Miragall *et al.*, 1992).

Neurons in arthropods have long been known to use electric synapses (gap junctions) for the passage of micro-currents (see Bennett, 1973). In addition to the G-protein mediated activity by neurotransmitters and hormones, levels of second messenger in the post-synaptic cells may be altered by their direct diffusion through gap junctional channels. Several hypothetical models of such controls are presented by De Mello (1990). Cx32 has been shown to be expressed in the neuronal subpopulations of the brain stem, mesencephalon, various cerebral cortical layers, hippocampus (in neonatal brain), and in basal ganglia (Dermietzel *et al.*, 1989; Micevych and Abelson, 1991; Matsumoto *et al.*, 1991).

During embryonic development, both the pattern and type of connexin expression is modulated. The presence of Cx26 has been reported in embryonic brain as early as the

E12 stage, whereas Cx32 is completely absent and only starts to appear postnatally. Connexin43 mRNA is readily detected in rat and mouse brain at birth, but Cx32 is barely detectable at this stage. However, by postnatal days 10 to 16, a sharp increase occurs in Cx32 mRNA (Belliveau *et al.*, 1991)

Regulation of gap junctions

Gap junctions are under the control of multiple mechanisms which can lead to an increase or reduction of intercellular communication with a wide spectrum of time courses, i.e.; from milliseconds to hours (reviewed by Loewenstein, 1981; Spray and Bennett, 1985; Warner, 1988; Sáez *et al.*, 1993; Bennett *et al.*, 1991). In principle, gap junctional communication can be regulated at the levels of cell-cell adhesion, connexin gene transcription and translation, connexin assembly, connexin degradation/reutilization, and opening and closing of the channels (Musil and Goodenough, 1990). The modulation of gap junctions traditionally has been studied by the passage of a current between the coupled cells. However, the microinjection of tracers such as membrane impermeable fluorescent dyes, and more recently, changes in connexin mRNA and proteins have been assayed for gap junctional regulation. Some major modulators of gap junctional communication and gene regulators are discussed below.

Calcium ions

Délèze (1964) noticed that 'healing-over' of cardiac cells after physical injury in which healthy cells isolate themselves from their injured neighbors occurs only if the extracellular medium contains calcium ions. This provided an insight into a role of calcium ions in gap junction channel occlusion (see Peracchia, 1987). The relationship between calcium ions and gap junction uncoupling was shown by intracellular injections of Ca^{++} in gap junction channel closure. When the intracellular concentration of free calcium ions rises, gap junctions close (Loewenstein, 1966; Nakas *et al.*, 1966; Rose and Loewenstein, 1976).

Unwin and Ennis (1984) have demonstrated that a reversible and co-ordinated rearrangement of the connexin subunits around the channel are induced by Ca^{++} that causes closure of the channels. Gap junction channels are sensitive to Ca^{++} in the low micromolar range or less, but higher concentrations (10-100 μM) are necessary to close all the channels and cause complete cell-to-cell uncoupling (Peracchia, 1987). The calcium

sensitivity of gap junctional conductance varies in different tissues as junctions in some tissues are sensitive to even physiological Ca^{++} levels (Noma and Tsuboi, 1987) whereas others are much less sensitive (Spray *et al.*, 1982). The sensitivity of the junctions to calcium ions depends on the ambient pH; as the pH falls, the junctions becomes less sensitive to a rise in Ca^{++} (Warner, 1988). In some tissues it has been shown that lowering of intracellular pH causes an elevation of Ca^{++} which in turn causes channel closure (discussed by Loewenstein, 1981). However, this phenomenon is not seen in other tissues, for example, in fish and amphibian embryos, it was shown that lowering of intracellular pH by exposure to CO2 could cause uncoupling of junctions without much change in the Ca^{++} (Bennett, *et al.*, 1978; also see review by Spray and Bennett, 1985).

The effects of intracellular pH and Ca^{++} on channel gating has been shown to be mediated by a diffusable cytoplasmic molecule, because after internal perfusion of cells Ca^{++} or H^{+} ions failed to affect junctional permeability (Johnston and Ramón, 1982; Arellano *et al.*, 1986). Calmodulin is an obvious candidate that could mediate the effects of intracellular calcium ions in channel gating because addition of calmodulin in the perfusate rendered the channels sensitive to Ca^{++}. Calmodulin is required for Ca^{++}-induced reduction of channel permeability (Girsch and Peracchia, 1985; Peracchia and Girsch, 1985). Thus, a number of studies indicate that intracellular calcium plays an important role in gap junction channel opening and closing; however, in a recent review Sáez *et al.* (1993) have discussed the possibility that intracellular calcium in the physiological range may not inhibit channel communication.

Intracellular pH

An uncoupling effect in parallel with a decrease in intracellular pH was first reported by Turin and Warner (1977; 1980) in *Xenopus* embryos; lowering of the intracellular pH below 7 resulted in a substantial reduction of gap junctional coupling. Spray *et al.* (1981) also demonstrated a role for intracellular pH, but not of extracellular pH, on junctional channel conductance. It is not well characterized how a change in intracellular pH can regulate the opening or closing of channels. It is possible that change in intracellular pH is secondary to other regulatory mechanisms such as the aforementioned rise in the intracellular calcium.

Although the buffering power of cytoplasm is considerable at normal intracellular pH, and fluctuations in gap junctional conductance during normal physiological processes

are likely to be small, pathological conditions may cause large changes in pH, and thereby affect the gating function of gap junctions (De Mello, 1987)

Cyclic AMP

Agents that elevate cAMP increase junctional conductance in hepatocytes, which express Cx32 and Cx26, and in cardiac myocytes which express Cx43 (Sáez *et al.*, 1986; Burt and Spray, 1988). Cyclic AMP may also have inhibitory effects on junctional conductance; in uterine muscle which expresses Cx43, cAMP decreases coupling (Cole and Garfield, 1986). These changes are rapid, occurring within minutes suggesting an effect on gating. However, long term effects of cAMP over a few hours, result in the enhancement of transcription or translation of connexin (Kessler *et al.*, 1984; Azarina *et al.*, 1981; Traub *et al.*, 1987). It appears that cell-specific pathways of signal transduction are responsible for such opposing actions of cAMP in different systems (Bennett *et al.*, 1991).

Tumor promoters

Several different tumor promoters have been studied for their effect on gap junctional intercellular communication and on connexin expression. All the tumor promoters tested in these studies cause a transient decrease in gap junctional communication and also reduce the expression of connexin.

Most of these studies have been carried out *in vitro* and involve cells expressing Cx32, Cx26, and Cx43. The tumor promoters tested are TPA (Brissett *et al.*, 1991; Oh *et al.*, 1991), DDT (Rivedal *et al.*, 1994), dieldrin, heptachlor epoxide (Matesic *et al.*, 1994), BHT (Chaudhuri *et al.*, 1993) and endosulfan (Kenne *et al.*, 1994). *In vivo* administration of the liver tumor promoting agent DDT in rats inhibited hepatic gap junctional communication in a dose-dependent manner and caused aberrant Cx32 and Cx26 expression (Tateno *et al.*, 1994). Although the mechanism of inhibition of gap junctional communication appeared to be at the level of phosphorylation of existing connexins, there were reported differences on the degree phosphorylation of connexin molecules by different agents. Some tumor promoters such as TPA resulted in 'hyperphosphorylation' of connexins, whereas dieldrin and heptachlor epoxide caused 'hypophosphorylation' (Matesic *et al.*, 1994) of Cx43 in the WB-F344 rat epithelial cell line. In another study on IAR20 rat liver epithelial cells, TPA caused marked hyperphosphorylation of Cx43, whereas the chlorinated insecticide endosulfan only

slightly increased phosphorylation initially followed by hypophosphorylation (Kenne *et al.*, 1994). The foregoing indicates that different tumor promoters use different signaling pathways to affect the gap junctional communication.

Hormones

Several growth factors and hormones have been studied for their effects on connexin regulation. Table 2.3 lists some of these factors and their effects on connexins in different types of cells. Although the endocrine hormones appear to affect the long term regulation of connexins by modulating transcription, cell growth factors probably affect the short term effects through phosphorylation.

A causal relationship between hormonal levels in the body and the appearance of gap junctions has been observed in pregnant human females. Smooth muscle cells in the non-gravid uterus are weakly interconnected through gap junctions, but 24 hours prior to parturition, and in response to circulating hormones, there is an explosive increase in gap junctional communication, which is accompanied by the formation of large numbers of gap junctions between myometrial cells (Garfield *et al.*, 1977; 1978), and increased *de novo* synthesis of the Cx43 (Risek *et al.*, 1990; Garfield *et al.*, 1980; Tabb *et al.*, 1992; Chow and Lye, 1994). Intercellular communication is rapidly lost within a day after parturition. The foregoing increase in myometrial gap junctions can be mimicked by administering estrogen, whereas progesterone inhibits gap junction formation (Cole and Garfield, 1985). Direct enhancement of Cx43 by estrogen administration and inhibition by progesterone have been demonstrated (Petrocelli and Lye, 1993). Additionally, when Cx43 promoter-luciferase reporter gene and estrogen were co-expressed in transfected cells, luciferase was expressed in the cells on exposure to estrogen (Yu *et al.*, 1994). This clearly shows that at least Cx43 gene expression is responsive to estrogen.

Hormones also are known to regulate connexin expression in the ovarian follicle. Gap junctions between oocytes and follicle cells occur in *Xenopus* (Brown *et al.*, 1979) and in mammals (Gilula *et al.*, 1992). These junctions are regulated by gonadotropins. Upregulation of gap junctional communication has been reported in Sertoli cells grown in culture grown after treatment with FSH (Pluciennik *et al.*, 1994).

Table. 2.3. Factors affecting the connexin expression and gap junctional intercellular communication in different cells and tissues.

Factor	Tissue/Cell	Effect on Cx level/GJIC	Reference
EGF	HUVEC	Decrease	Xie & Hu, 1994
bFGF	BME cells	Increase	Pepper & Meda, 1992; Troyanovsky et al., 1993
TGFβ	Mammary tumor cell line	No effect	Lee et al., 1992
IL-1	Bone marrow stromal cells	Decrease	Dorshkind et al., 1993
PDGF-BB	Mouse fibroblast cell line C3H/10T1/2	Decrease	Pelletier et al., 1994
TSH	Thyroid epithelial cell culture	Increase	Munari-Silem et al., 1994
FSH	Sertoli cells	Increase	Pluciennik et al., 1994
Gonadotropins	Ovarian follicle	Decrease	Schrieber et al., 1993
Androgen	SNB motoneuron	Increase	Matsumoto et al., 1991
ACTH	Sheep	Increase	McNutt & Nicholson, 1994
Estrogen/17-β-estradiol	Rat myometrium	Increase	Petrocelli and Lye, 1993
Estrogen	Ovarian follicle	Increase	Schrieber et al., 1993
Progesterone	Rat myometrium	Decrease	Petrocelli and Lye, 1993
PTH	Rat osteoblasts	Increase	Schiller et al., 1992
Glucocorticoids (Dexamethasone)	Rat hepatocytes cell cultures	Increase	Kwiatkowski et al., 1994
Retinoids	C3H 10T1/2 cells	Increase	Rogers et al., 1990
Endothelin	Mouse primary astrocytes	Decrease	Giaume et al., 1992
Myotrophin	Cardiac myocytes	Increase	Mukherjee et al., 1993
Norepinephrine	Rat pinealocyte cultures	Increase	Saez et al., 1991

Phosphorylation of connexins

The normal functioning of the gap junctions requires the specific phosphorylation of connexins (Musil *et al.*, 1990 a; 1990 b; Musil and Goodenough, 1991). Phosphorylation plays a role in gap junction assembly, channel opening and closing, and connexin degradation (Musil and Goodenough, 1990). Agents such as tumor promoting phorbol esters that modulate intracellular kinase pathways also have been found to modulate gap junctional communication. These effects are usually seen within a few minutes, indicating a direct effect on the phosphorylation of existing proteins.

Inhibition of junctional communication occurs through different phosphorylation pathways. Tumor promoter TPA caused inhibition of gap junctional communication by phosphorylation on the serine residues (Brissett *et al.*, 1991) whereas viral oncogenes $pp60^{v\text{-}src}$ caused phosphorylation of tyrosine residues on connexin43 (Crow *et al.*, 1992). It appears that the diversity of effects on gap junctions results from the involvement of intermediates in the signal transduction pathway which determine the overall response. Such intermediates may act in a cell-specific manner and allow each cell to set the functional state of its gap junctions in response to prevailing external and internal conditions (Münster and Weingart, 1993).

Functions of gap junctions

Despite the impressive progress made in knowledge about the molecular structure, gene cloning, *in vitro* mutagenesis, and spatial and temporal distribution of gap junctions, very little is known about the precise function(s) of gap junctions in cellular and tissue physiology. A number of functions have been ascribed to gap junctions which are discussed below, but it is important to consider that these are based on indirect evidence.

Tissue homeostasis and molecular co-operation

The most basic physiological role of the gap junction channel is homeostatic: a buffering of individual variations in channel-permeant molecules in tissue cells (Loewenstein, 1966; Ledbetter and Lubin, 1979; Larson, 1990). At the most fundamental level, coupled cell populations can remain autonomous with respect to macromolecules but interdependent with respect to small metabolites (Sheridan and

Atkinson, 1985). In cells remote from blood supply and where the paracellular diffusion route is tighter, the gap junction channels may be a secondary pathway or even a primary route of transport to cells that have no access to the extracellular fluid, such as the lens and myelin sheath formed by Schwann cells (Loewenstein, 1981).

Metabolic co-operation between $HPRT^-$ mutants and $HPRT^+$ wild type cells was demonstrated by Subak-Sharpe *et al.* (1969). The wild type cells utilize hypoxanthine in their nucleotide pool in nucleotide free media whereas the mutants cannot. In co-cultures of mutants and wild type cells it was found that hypoxanthine was incorporated into mutant cells which was transferred from wild type cells via gap junctions. It was also found that mutant cells 'stimulated' the wild type cells to produce more nucleotides (discussed by Sheridan and Atkinson, 1985).

Electrical conduction and synchronization of activity

In excitable cells, ionic coupling is responsible for a functional electrical synapse or electrotonic junction (Bennett, 1973; Bennett and Verselis, 1992). These occur where nerve or muscle cells must respond in rapid synchrony. Electrical signal transduction in a non-nervous tissue is best exemplified by myocardium. Escape systems involving rapid movements, and synchronization of neuronal firing are other examples where electrical transmission is common. Electrical transmission is very rapid because it does not involve the delay associated with transmitter release (Bennett, 1973). In non-excitable cells, the functions of ionic coupling remains unclear.

The myometrial gap junction protein Cx43 is thought to be critical to the development of synchronous, high-amplitude contractions of the myometrium during labor (McNutt *et al.*, 1994). Measurement of the electrical activity of the uterus, similar to that of heart, is being tested by Garfield and co-workers in animals and humans to provide diagnostics for labor induction (Radestsky, 1994). This could be useful in preventing premature labor and delivery.

Transfer of signals

Gap junctional communication appears to be such a universal phenomenon that it is obvious that it must be integrating a number of pathways, primarily through the dispersal of small molecules. However, it is becoming increasingly apparent that the

cellular processes occur in more or less defined intracellular compartments, and it therefore seems unlikely that gap junctions could drastically affect the basic metabolism of cells, unless the reaction in question is dependent upon direct cell to cell molecular transfer (seen as metabolic cooperation in some cases). The transmission of signaling molecules could be a more useful role for gap junctions. Although it is known that cells do affect their neighbors via intercellular channels, the identity of the signaling molecules remains speculative (see Sheridan and Atkinson, 1985). Lawrence *et al.* (1978) demonstrated the passage of second messengers via gap junctions in co-cultures of heart and ovarian-granulosa cells, which are responsive to noradrenalin and FSH, respectively. Treatment with either agent caused functional changes in the other cell type. These experiments demonstrated the involvement of diffusable factors which pass through gap junction channels and elicit responses in coupled cells. Second messenger molecules such as Ca^{++}, cAMP, and inositol phosphate can pass through gap junction channels and they may cause physiological changes in connected cells (Sáez *et al.*, 1989; Enkvist and McCarthy, 1992). The effects of these molecules, however, may well dependent on the physiological state of the recipient cell, as determined by stage of development, and environmental or hormonal influences.

The intercellular spread of calcium waves is mediated by gap junctions (Charles *et al.*, 1992; Enkvist and McCarthy, 1992). Mechanical stimulation of a single cell in a Cx43 synthesizing transfected C6 rat glioma culture induced a calcium wave that was communicated to neighboring cells via gap junctions. By contrast in untransfected cells which did not show appreciable gap junctional communication the rise in intracellular calcium concentration was confined to mechanically induced cells (Charles *et al.*, 1992). The spread of lucifer yellow and calcium waves was inhibited if the cells were treated with either phorbol 12-myristate 13-acetate (PMA) or a synthetic diacylglycerol that activates protein kinase C (Enkvist and McCarthy, 1992).

Embryonic Development

Correlative evidence suggests that changes in the temporal and spatial patterns of gap junctions parallel the progressive determination of cell fate (Kolb and Somogyi, 1991). Evidence that links gap junctions with events in embryonic development has been reviewed (Bennett *et al.*, 1981; Guthrie, 1987; Guthrie and Gilula, 1989; Gilula *et al.*, 1992; Sáez *et al.*, 1993). The timing of the appearance, presence and disappearance of gap junctions precedes, or coincides with specific developmental events, which lends

support to the idea that communication via cell-to-cell channels provides a specific pathway for intercellular signals of a transient nature (Welsch, 1990).

One speculation about the function of gap junctions is that they may facilitate the buildup of chemical gradients during development (Warner and Lawrence, 1973; see reviews by Lawrence, 1981; Caveney, 1985; Warner, 1992). Formation of regional patterns of junctional communication among the cells were observed during *Xenopus* embryonic development. Cells from these 'communication compartments' displayed gap junctional coupling with one another, whereas dye coupling was restricted between compartments (Kalimi and Lo, 1988). This restriction of intercellular communication through gap junction closure or disappearance is thought to generate boundaries that might be necessary for cell differentiation (Sáez *et al.*, 1993). The first direct role for gap junctions in *Xenopus* development was demonstrated by Warner *et al.* (1984). Antibodies which completely but transiently blocked gap junctions caused defects in pattern formation. A similar role of gap junctions in chick embryo limb development also has been shown (Allen *et al.*, 1990).

Lawrence Katz and co-workers have shown that in developing mammalian brain, cortical neurons communicate via gap junctions to synchronize their activities (Peinado *et al.*, 1993; also see Nowak, 1992). Developmental regulation of gap junctional communication is also suggested by experiments in which the injection of RNA of the proto-oncogene wnt-1, (thought to be important for embryonic pattern formation), increases gap junctional communication in the ventral side of the *Xenopus* embryo (Olson *et al.*, 1991). However, not all processes in embryonic development require gap junctions. In *Xenopus,* the induction of muscle cell genes in mesodermal cells by vegetal pole cells proceeds unimpaired even when communication through gap junctions was completely inhibited (Warner and Gurdon, 1987). In a recent report in "knock-out" mice by targeted mutagenesis of connexin43, Reaume *et al.* (1995) showed that the lack of connexin in mouse embryos did not prevent the embryonic development, but cardiac malformation resulted in neonatal mice.

Pathological conditions

Different connexins confer specific electrophysiologic characteristics on assembled channel proteins. Reduction in Cx43 content is a general pathogenetic feature of cardiac disease, and changes in the expression levels of other connexin types may contribute to

altered electrophysiologic function in the diseased heart (reviewed by Severs, 1994). A marked disruption in the normal pattern of the gap junction network also has been reported in the vicinity of cardiac infarcts which might be a cause of inhomogeneous and slowed myocardial conduction (Green and Severs, 1993).

Recently, mutations in connexin32 have been correlated with X-linked Charcot-Marie Tooth Disease (CMTX), which is a form of hereditary neuropathy with demyelination (Bergoffen *et al.*, 1993 a, 1993 b). Direct sequencing of the Cx32 gene showed seven different mutations in affected persons from eight CMTX families. Also, from targeted mutagenesis study in connexin43 gene of mice (Reaume *et al.*, 1995), raised the possibility that some congenital heart abnormalties in humans may be due to loss or mutation in connexin43 (Stone, 1995).

Growth and Oncogenesis

Another postulated role of junctional transfer has been in the control of cell growth (reviewed by Bennett, 1973; Loewenstein, 1979, 1981; Sheridan, 1987; Bennett *et al.*, 1991; Loewenstein and Rose, 1992). It was proposed that the loss of growth control, a characteristic of malignancy, might be caused or contributed to by a loss of gap junctions (Furshpan and Potter, 1968; Loewenstein, 1979). This hypothesis was based primarily on two types of observations; first, cell growth is arrested in cultures when they reach confluency and form numerous gap junctions, and second, many tumors are not junctionally connected and their cells grow at faster rates than normal cells. It was proposed that soluble growth controlling factors may pass through gap junctions to exert control over the growth characteristics of the communicating cells. This hypothesis has drawn support from the findings that viral transformation of cells, and that treatment with tumor promoters lead to the closure and downregulation of gap junctions (see review by Sheridan, 1987; Trosko *et al.*, 1990; Loewenstein and Rose, 1992). Also, anti-tumor agents such as retinoids and carotenoids are known to enhance gap junctional communication in transformed cells (Guo *et al.*, 1992; Asato *et al.*, 1993; Bertram, 1993).

Using subtractive hybridization technique Cx26 was found to be a cellular component of normal cells but not of their transformed counterparts (Lee *et al.*, 1992). Experimental increase of Cx43 in the C6 rat glioma cell line by transfection of connexin43 cDNA has been shown to cause retardation in the rate of cell growth (Naus *et al.*, 1991).

However, this retardation is mediated through secreted factors, and not the factors that pass directly through the channels (Zhu *et al.*, 1992) because the medium conditioned by connexin43-overexpressing C6 cells repressed the growth of non-transfected C6 cells.

Many transformed cells and tumors form gap junctions and communicate through these channels. Different cells that communicate via gap junctional channels grow at different rates in tissues *in situ*. Induction of oncogenesis and growth are directly under the control of tumor-suppressor genes, growth factors, growth factor receptors, and their respective signaling pathways (Rozengurt, 1992; Baserga, 1994). The proposed role of gap junctional communication in the control of cellular growth depends on correlative data and direct evidence for this possible function is still lacking.

Cell cycle and factors affecting cell proliferation

Uncontrolled cell growth is a characteristic of cancerous cells. Loss of gap junctions is thought to be a factor in tumorigenesis. However, transformation of normal cells into cancer cells is a complex multistep process, and cancer cells differ from normal cells in many important characteristics including increased cellular growth, loss of differentiation, increased invasiveness, and decreased drug sensitivity. These differences do not arise simply from impaired growth control. Multiple genetic changes occur during the transformation of normal cells into cancer cells, and this transformation is facilitated by loss of fidelity in the processes that replicate, repair, and segregate the genome (Hartwell and Kastan, 1994). Chromosomal abnormalities in human tumors most often include the creation of tumor-specific fusion proteins or translocations resulting in activation of proto-oncogene products (Rabbitts, 1994); these gene products are generally transcription factors.

Since the demonstration that v-*sis* transforming oncogene is homologous to the sequence of platelet-derived growth factor (PDGF) (Doolittle *et al.*, 1983; Waterfield *et al.*, 1983), several cell-derived oncogene products have been found to be homologues of growth factors, of growth factor receptors, or of molecules on the signal-transducing pathways of these receptors (Baserga, 1994).

Cell proliferation is essentially the overt manifestation of the pace of the cell cycle and of the number of cells actively dividing in a population. The cell cycle is controlled by different cyclins, cyclin-dependent kinases (CDK), and their inhibitors (CDI) (reviewed

by Sager, 1992; Rozengurt, 1992; Hunter and Pines, 1994; Baserga, 1994). The proliferation of most cells can be stimulated by a variety of extracellular ligands that act in a combinatorial and synergistic fashion. These factors bind to receptors located on the cell surface and activate multiple signaling pathways including those involving tyrosine kinases and G-protein mediated pathways (Rozengurt, 1992). These pathways directly or indirectly affect the cell cycle. The cell cycle is regulated at checkpoints, and many of these checkpoints are deregulated in oncogenesis, which in turn is mainly due to changes in cyclin-CDK complexes (Hunter and Pines, 1994). Overexpression of positive regulators (cyclins) or loss of negative regulators (inhibitors of cyclin dependent kinase, CDI) may cause these deregulations. The increase in proliferative activity by the deregulation of cyclins may not be sufficient to neoplastically transform cells; it may co-operate with other proto-oncogenes, such as Ras in this type of transformation (discussed by Hunter and Pines, 1994).

Tumor suppressor genes are recognized to serve regulatory functions by counteracting the proto-oncogene products that drive proliferation and other cancer-promoting activities (Sager, 1992). Again, the interactions between the products of proto-oncogenes and tumor suppressor genes may be mediated by cyclin dependent kinases, as exemplified by the retinoblastoma tumor suppressor gene Rb, and p53 (discussed by Hall, 1991; Sager, 1992).

Other cytoplasmic factors that can control the growth of cells also have been identified. Two proteins, namely statin and terminin, have been identified in cultured fibroblasts and in liver tissues (see review by Wang, 1992). Statin is found only in the nucleus of non-proliferating cells. Statin is present in quiescent and senescent cells whereas terminin is expressed only in senescent cells. Nuell *et al.* (1991) also have reported a protein called prohibitin which is involved in growth arrest. Microinjection of synthetic prohibitin mRNA into cells blocks their entry into the S phase of the cell cycle.

The role of membrane K^+ channels in mitogenesis also has been reported (for review see Dubois and Rouzaire-Dubois (1993). Many antitumor agents have been found to block K^+ channels. The initiation of cell division, following interaction with their receptors, is characterized by a complex series of molecular events including transient changes in membrane permeability to ions. In neuroblastoma cells, inhibition of proliferation by tamoxifen is also associated with closure of K^+ channels (Rouzaire-Dubois and Dubois, 1990, 1991). Tamoxifen also inhibits the proliferative activity of

human malignant gliomas (Pollack, 1990). It remains to be determined whether the correlation between K^+ channel activity and cell proliferation is direct or fortuitous.

Chapter 3

MATERIALS AND METHODS

To achieve the objectives outlined in the Chapter 1, various materials and methods were used in this study. These materials and methods are described in the following sections.

Plasmids

Plasmid constructs containing cDNA of full length Cx43, Cx32, and antisense Cx43 were obtained from the laboratory of Dr. Nalin M. Kumar and Dr. Norton B. Gilula, The Scripps Research Institute, La Jolla, USA. Figure 3.1 shows the diagram of the plasmid pT7/T3 291 that contains a full length cDNA of Cx43 at the *EcoRI* site in the multiple cloning site of the vector. The total length of this cDNA is 2366 base pairs (bp). The Cx43 cDNA insert is flanked by viral transcription promoters T7 and T3 to synthesize the sense strand and the antisense strand, respectively. The antisense RNA synthesizing plasmid pNuTBGHE 914 (Fig. 3.2) contains a *HindIII* fragment of Cx43 (~1.6 kb) in antisense direction to the mouse metallothionein promoter, with a polyadenylation sequence downstream.

Antibodies

Affinity purified polyclonal antibodies to Cx43 were obtained from the laboratory of Drs. Nalin M. Kumar and Norton B. Gilula. These antibodies were directed to a synthetic carboxyl-terminus peptide sequence of Cx43 (Risek *et al.*, 1992) and are designated as α_1s. These antibodies have been characterized for their specificity to the Cx43 protein by immunocytochemistry and by Western blotting (Yeager and Gilula, 1992). On Western blots, these antibodies recognize both phosphorylated and non-phosphorylated species of Cx43 protein.

Cell culture

Rat glioma cell lines C6 (ATCC CCL 107) and 9L (obtained from Dr. D. Boisvert, Department of Surgery, University of Alberta) were used for this study. Both cell lines

Figure.3.1. Diagram showing the relevant parts of plasmid T7/T3 291. The connexin43 cDNA of 2366 base pairs is inserted at the *EcoRI* site within the multiple cloning site of plasmid T7/T3α-19 (Gibco-BRL, Canada). The connexin43 cDNA is flanked by T3 and T7 promoters which can be used for sense or antisense RNA probe synthesis. The sense RNA probes contained the sequence upto *SacI* (539) site, whereas the antisense RNA probes contained the sequence from end to *AflIII* (2185) site.

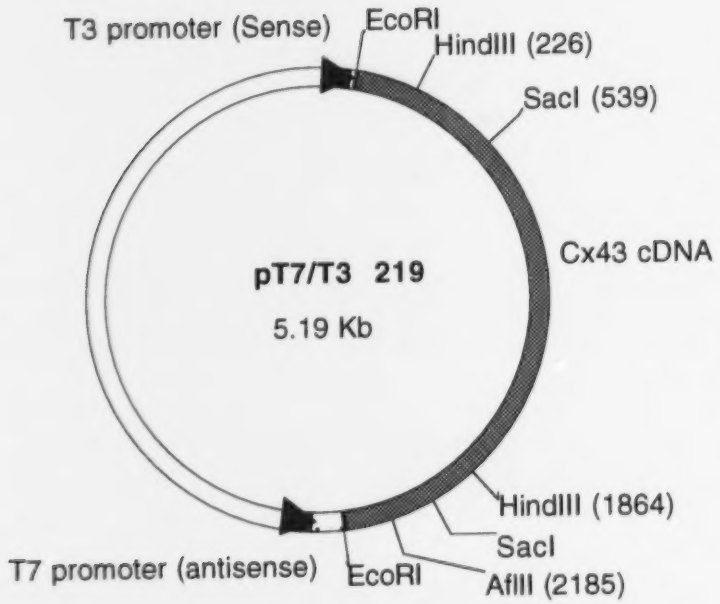
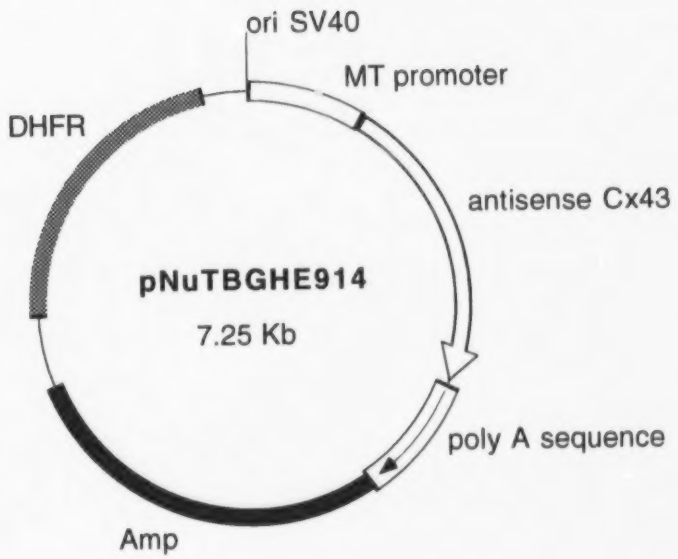


Figure.3.2. Map of antisense connexin43 RNA synthesizing plasmid pNuTBGHE 914. The size of the plasmid is approximately 7.25 kb. The locations of mouse metallothionein promoter and cDNA are marked. The plasmid also contains sequences for ampicillin resistance for maintenance in *E. coli* and a eukaryotic selection marker dihydrofolate reductase (DHFR).



were originally induced with methylnitrosourea (Benda *et al.*, 1968; 1971). The C6 and 9L cells are classified as glioblastoma and gliosarcoma, respectively (reviewed in Cravioto, 1986). Cell lines 9L and C6 were maintained in 25 cm^2 flasks under standard laboratory conditions (37°C, 5% CO_2) in Eagle's minimal essential medium (MEM, Gibco, Canada) supplemented with 10% fetal calf serum, 2 mM glutamine, and 100 units penicillin/ 100 µg/ml streptomycin (Gibco-BRL, Canada). Cells were grown on coverglasses for experiments.

Transfection of cells

The 9L cells were inoculated in 35 mm plastic petri-dishes at a density of approximately 10^5 cells per petri-dish containing 2 ml growth medium (MEM containing 10% fetal bovine serum, 100 units/ml penicillin and 100 µg/ml streptomycin, and 2 mM glutamine). After the cells had spread on the bottom, transfection was done using the Lipofectin reagent (Gibco BRL, Canada) according to the manufacturer's protocol described below.

A plasmid DNA construct of antisense connexin cDNA (pNuTBGHE 914) was mixed with the plasmid pSFFVneo (which contained the neomycin resistance marker) in a three to one molar ratio. The plasmids in the mix were precipitated, washed with 80% ethanol and redissolved in 100 µl sterile double distilled water. A 50 µl aliquot of these plasmids was mixed with an equal volume of Opti-MEM-I low serum medium (Gibco BRL, Canada). A 10% (v/v) dilution of Lipofectin reagent (Gibco BRL, Canada) in MEM was added to each DNA sample (100 µl to each). After incubation for 15 minutes at room temperature, 1.8 ml serum-free medium was added to each DNA sample. This mixture was then poured into petri-dishes containing 9L cells and placed in an incubator (37°C, 90% humidity, and 5% CO_2).

After 8 to 12 hr of incubation in the presence of extraneous DNA, the cells were washed with MEM (Gibco-BRL, Canada) and complete medium was added. Two days later, the cells were trypsinized (0.5% trypsin, 1mM EDTA in PBS) and plated on two 100 mm petri-dishes in the selective medium which contained the growth medium plus geneticin antibiotic (G418; 270 µg per ml of medium).

The medium was changed at 4 day intervals, and after 15 days in the selection medium, the G418-resistant cell colonies were isolated using cloning cylinders. Cloning

cylinders were made from plastic 1 ml pipette-tips by cutting the collar with a razor blade. One end of these cloning cylinders was coated with sterile vacuum grease and the cylinders were placed around the cell colonies on the petri-dishes from which the growth medium was removed. A 200 µl aliquot of trypsin solution was added to each cylinder to detach the cells from the dishes and the cells were transferred to petri-dishes containing fresh growth medium. These clones were marked and grown in separate cultures and kept frozen in liquid nitrogen.

Growth curve of cells

For comparison of the cell growth rates of the 9L and C6 cell lines, cells were plated in triplicates in 25 cm^2 plastic culture flasks. Each flask was seeded with 10^5 cells in 5 ml of growth medium. Three flasks of each cell type were trypsinized (0.1% trypsin, 2 mM EDTA in PBS), at daily interval. Dilutions of triplicate cultures were mixed with Trypan blue vital dye (0.1% w/v final concentration in phosphate buffered saline, PBS = 137 mM NaCl, 2.7 mM KCl, 10.6 mM Na_2HPO_4 and 1.5 mM KH_2PO_4), and counted in a hemocytometer. Doubling times were calculated based on viable cell counts obtained during the log growth phase, and graphs were plotted (Freshney, 1994). The growth rates of transfected and untransfected 9L cells were determined both in the presence and absence of 70 µM zinc acetate in the growth medium. Cells were plated in triplicates in 35 mm petri-dishes containing 2 ml of medium. At daily intervals, three plates of each cell type were trypsinized and counted using a hemocytometer. Statistical analyses were done using SPSS for Windows.

Electron microscopy

For conventional electron microscopy, 9L and C6 cells were grown on glass coverslips as described above, and fixed in 2% glutaraldehyde in cacodylate buffer (0.1 M, pH 7.2) containing 1% colloidal lanthanum hydroxide prepared as described below. After washes in cacodylate buffer, secondary fixation was carried out in 2% aqueous osmium tetroxide for 1 hour at room temperature. Then the cells were dehydrated through a graded ascending series of alcohol (using 10% to 98% ethanol). Following dehydration, the cells were passed in succession through propylene oxide at 100% v/v, 25% Epon and propylene oxide, 50% Epon in propylene oxide, and then embedded by inverting Epon-filled embedding capsules over the coverslips. Ultrathin sections were cut on a Reichert ultra-microtome, stained with saturated aqueous uranyl acetate and lead citrate, and

examined by transmission electron microscopy either on Philips 400 or Philips 201 electron microscope.

Preparation of lanthanum hydroxide

Colloidal lanthanum hydroxide was prepared by the method described by Revel and Karnovsky (1967). A 1% aqueous solution of lanthanum nitrate was brought to pH 7.6 by slowly adding 0.01 N sodium hydroxide solution with vigorous stirring. A light flocculent colloidal suspension was obtained which was added to the fixative to a final concentration of 0.5%.

Immunofluorescence labeling

For immunofluorescence labeling, cells were grown on glass coverslips, and fixed either in methanol for 5 minutes at -20°C or in freshly prepared 4% paraformaldehyde for 20 minutes at room temperature. The coverslips were then washed in phosphate buffered saline (PBS, 137 mM NaCl, 2.7 mM KCl, 10.6 mM Na_2HPO_4 and 1.5 mM KH_2PO_4). For blocking non-specific labeling, the cells were incubated for 1 hour at room temperature in 3% bovine serum albumin and 1% normal goat serum in PBS. The blocking solution was removed and antibodies either to Cx43 or pre-immune serum were added onto the coverslips at 1:50 or 1:100 dilution in PBS. After incubation for 2 hours at room temperature with the primary antibody or with pre-immune serum, the coverslips were washed three times in PBS and incubated with a 1:100 dilution of goat-anti-rabbit IgG conjugated to FITC (1 hour, 37°C). Finally, the coverslips were washed three times with PBS, then once with double distilled water, and mounted in glycerol/p-phenylenediamine. Prepared slides were examined either by epifluorescence microscopy (Zeiss, Germany) using appropriate filters or by laser confocal microscopy (Molecular Dynamics, USA).

Scrape loading/Dye transfer

Intercellular spreading of the fluorescent dye Lucifer Yellow CH was monitored by the scrape loading/dye transfer method described by El-Fouly *et al.* (1987). Cells were grown on glass coverslips to confluency either in 35 mm plastic petri-dishes or in 6 well plates. Growth medium was removed and after a rinse with a salt solution (116.36 mM NaCl, 5.4 mM KCl, 0.82 mM $MgSO_4.7H_2O$, 10.15 mM NaH_2PO_4, 25 mM $NaHCO_3$,

and 25 mM HEPES, pH 7.4), Lucifer Yellow CH solution (0.5 mg/ml, Sigma, USA) was added to the dishes. Linear scrapes across the surface of each coverslip were made with a sharp razor blade. After a two minute exposure, the dye solution was removed and coverslips were washed three times with salt solution. Each wash was for 2 minutes. Cells were then fixed with 4% paraformaldehyde for 15 minutes at room temperature and washed with salt solution. Coverslips were mounted in p-phenylenediamine-glycerol. The slides were viewed by epifluorescence microscopy (Zeiss, Germany).

Immunoblotting

Sample preparations

Protein samples for immunoblotting were prepared either as crude homogenates of the cells or as crude alkali preparations of gap junctions. For crude homogenates, cells grown on plastic petri-dishes were rinsed with PBS and scraped in 1x Laemmli's buffer (Laemmli, 1970). Alternatively, cells were first scraped in gap junction solution (1 mM sodium bicarbonate, pH 7.0, 1 mM PMSF, and phosphatase inhibitors) and then mixed with equal volumes of 2x Laemmli's buffer without β-mercaptoethanol. The samples were passed through a 23 G needle several times to break up the cells. Preparation of the crude gap junction fraction (alkali insoluble fractions) was done according to the method described by Brissette *et al.* (1991). Briefly, cells were rinsed with PBS, scraped with gap junction solution, and mixed with an equal volume of freshly prepared 4 mM NaOH. These samples were kept on ice for 30 minutes with occasional mixing and centrifuged at 10,000 Xg for 30 minutes at 4°C. The pellets thus obtained were rinsed once with the gap junction solution and then dissolved in 1 x Laemmli's buffer without β-mercaptoethanol. The protein contents in each sample was estimated using the BCA method (Pierce Co., USA). Prior to electrophoresis, β-mercaptoethanol was added to the samples, and samples were loaded on polyacrylamide gels either after heating in a boiling water bath (~90°C) or without heating.

Electrophoresis and blotting

Electrophoresis of protein samples was performed on 12% polyacryiamide gels in BioRad's Mini Gel Electrophoresis Apparatus. After electrophoresis, proteins were electrophoretically transferred to nitrocellulose membranes followed by overnight incubation in blocking buffer (5% skimmed milk powder in TBS + 0.1% Tween20) at

room temperature. After rinsing with wash buffer (0.1% Tween20 in TBS), blots were incubated with a 1: 400 dilution of Cx43 antibodies or with pre-immune serum (room temperature, 2 hours), then rinsed with wash buffer and incubated for 1 hour with a 1: 5000 dilution of secondary antibody (goat-anti-rabbit-IgG) conjugated to horseradish peroxidase (BioRad, USA). The location of bound secondary antibodies was detected with ECL reagent (Amersham, Canada).

Northern blotting

Isolation of RNA

Total cellular RNAs were isolated from the cultured cells by using the guanidinium/cesium chloride method described by MacDonald *et al.* (1987). Cells were scraped and disrupted in 7 ml of guanidinium solution (4 M guanidinium thiocyanate, 50 mM Tris.HCl, pH 7.6, 0.5% N-lauryl sarcosinate, 1% β-mercaptoethanol) using a Polytron. The homogenate was centrifuged at 8,000 X g to remove cell debris and loaded onto a cushion of 3.3 ml cesium chloride solution (5.7 M CsCl, 0.1 M EDTA) in polyallomer centrifuge tubes. The tubes were centrifuged at 30,000 rpm in a Beckman SW40Ti rotor (114,000 x g) for more than 24 hours to selectively pellet the RNA. RNA pellets were dissolved in DEPC-treated water and were spectrophotometrically quantitated by absorbence at 260 nm. Alternatively, the total RNA from cells was isolated using the isolation method of Chomczynski and Sacchi (1987). Cells in petri-dishes were rinsed with ice-cold PBS and scraped after adding 0.5 ml guanidinium thiocyanate solution (4 M guanidinium thiocyanate, 25 mM sodium citrate, pH 7.0, 0.1 M β-mercaptoehanol) directly to the culture dishes. One-tenth volume of 2M sodium acetate (pH 4.0), one fifth volume of 10% SDS, an equal volume of phenol and one-fifth volume of chloroform were added and tubes were vigorously shaken. After a 15 minute incubation on ice, tubes were centrifuged and the upper aqueous phase was re-extracted with phenol- chloroform after continuous agitation at room temperature for 10 minutes. RNA was precipitated at -20°C overnight by adding an equal volume of isopropanol, pelleted, and redissolved in guanidinium solution. Phenol-chloroform extraction and precipitation was repeated, and after an 80% ethanol rinse, the RNA pellet was dissolved in DEPC-treated water.

Electrophoresis and blotting of RNAs

The RNAs obtained were quantitated by their absorbence at 260 nm. Equal amounts of total RNA samples were denatured in 50% formamide, 6% formaldehyde, 1 x MOPS buffer (1 x MOPS Buffer =0.02 M MOPS, 5 mM NaOAc, and 1 mM EDTA) by heating at 65°-70°C for 10 minutes. Electrophoresis was done under denaturing conditions on a 1.5% agarose-formaldehyde gel. After electrophoresis, RNAs were capillary blotted onto Hybond C-super membrane (Amersham, USA) using 10 X SSC SSC (1 X SSC= 150 mM NaCl, 15 mM sodium citrate, pH 7.0); the blots were rinsed in 6 X SSC, air dried, and baked *in vacuo*.

Probe synthesis and hybridization

Probes for Cx43 RNA detection were synthesized either by nick-translation of cDNA or by synthesizing riboprobes from a plasmid construct containing the Cx43 cDNA sequence. Nick-translated probes were synthesized off a purified *HindIII*-fragment of plasmid pT7/T3 291 which contains about 1.6 kb sequence of Cx43 cDNA ranging from 226 to 1864 bp. For the probe synthesis by nick-translation, the reaction mixture contained 0.2 µg cDNA, 22 µM of each dATP, dTTP, and dGTP in Tris-HCl (pH 7.8), $MgCl_2$, and β-mercaptoethanol with 6 µl of [^{32}P]-dCTP (800 ci/mmol, NEN, USA) and 2 µl (1 unit) DNA polymerase I. The nick-translation reactions were done at 15°C for 1 hour. T7 RNA polymerase was used to synthesize a riboprobe from *AflIII* endonuclease digested plasmid pT7/T3 291 (see Fig. 3.1) in the presence of α-[^{32}P]-CTP. The riboprobe thus obtained was expected to be 236 nucleotides long, corresponding to nucleotides 2185 to 2361 of the Cx43, including 60 nucleotides of the vector. Mouse β-actin probes were synthesized from the DNA constructs obtained from Ambion Co. (USA).

RNA blots were prehybridized in bags containing a hybridization mix. The hybridization solutions and hybridization temperatures were different for nick-translated probes and for riboprobes. For riboprobes the hybridization mix contained 50% formamide, 27.5 mM PIPES, 750 mM NaCl, 25 mM EDTA, 1% SDS, and 5x Denhardt's solution, whereas for nick-translated probes the mix contained 50% formamide, 10 mM NaH_2PO_4 (pH 7.0), 4 X SSC, 0.1% SDS, 2 mM EDTA, and 4 X Denhardt's solution. Hybridization was allowed for more than 2 hours at 65°C for riboprobes, and at 42°C for nick-translated probes. Nick-translated probes were denatured by heating in a boiling

water bath for about 5 minutes, snap-cooled in a dry ice-ethanol slurry and added to the hybridization bags. Riboprobes were denatured by heating in a boiling water bath, and then added directly to the bags. Hybridization was done overnight in a water-bath shaker. After hybridization, the blots were washed to a final stringency of 0.1 X, 0.4% SDS at 65°C. Blots were then exposed at -80°C to Fuji X-Ray film (RX-50) sandwiched between two intensifying screens. Densitometry was carried out on the autoradiographs to obtain band intensity peaks. Areas under the peaks were measured by using a MOP-3 digital image analysis system (Carl Zeiss, Germany).

Southern blotting

Isolation of genomic DNA

Cells were grown on 100 mm plastic petri-dishes to confluency. Cells were rinsed twice with ice-cold PBS and after adding 0.5 ml digestion buffer (10 mM Tris-HCl, pH 8.0, 100 mM NaCl, 25 mM EDTA, and 0.1 mg per ml Proteinase K) each petri-dish was swirled to spread the buffer over the cells. The viscous lysate was collected with the help of a rubber scraper, and incubated in a 50°C water bath overnight. The clear lysate was extracted at least three times with phenol-chloroform followed by a chloroform and isoamyl alcohol extraction. The DNA was then precipitated by adding 3 M sodium acetate (pH 5.2) and ice-cold ethanol at the room temperature. The visible fibrous DNA precipitate was spooled off, rinsed with 80% ethanol, air dried, and dissolved in TE (10 mM Tris.HCl, 1 mM EDTA, pH 7.6).

Southern Blotting

The DNA samples from untransfected 9L cells and transfected AB5 and AC4 cells were digested with *PstI* endonuclease (Pharmacia, USA). After incubation overnight at 37°C, the digested DNA samples were extracted once each with phenol and chloroform and chloroform-isoamyl alcohol. The DNA was precipitated overnight at -20°C with sodium acetate and ethanol, pelleted, rinsed with 80% ethanol, and resuspended in TE buffer.

The digested DNA samples were subjected to electrophoresis overnight at 20 volts constant voltage on a 0.8% agarose-Tris-Borate-EDTA gel containing ethidium bromide. The gel then was sequentially soaked in 0.25 N HCl for 30 minutes,

denaturation solution (1.5 M NaCl, 0.5 M NaOH) for 30 minutes, and neutralizing solution (0.5 M Tris-HCl, pH 7.2, 1.5 M NaCl, 1 mM EDTA) for 45 minutes with agitation. DNA was blotted onto Hybond N^+ charged nylon membrane (Amersham, USA) for 4 to 5 hours by capillary transfer using 10 x SSC. The blot was rinsed in 6 x SSC, air dried, and baked at 80°C for 2.5 hours.

Probe synthesis and hybridization

DNA blots were prehybridized for more than 3 hours in the hybridization mix (10 mM NaH_2PO_4, pH 7.0, 0.1% SDS, 4 X SSC, 2 mM EDTA; 50% formamide, 4 X Denhardt's solution, and 100 µg per ml salmon sperm DNA). Radiolabeled (^{32}P-dCTP) Cx43 DNA probes were synthesized by the nick translation method using a 1.6 kb *HindIII* fragment of the Cx43 cDNA. Hybridization was carried out overnight at 45°C. After hybridization, the blots were rinsed in 2 x SSC-0.1% SDS and then washed to a final stringency of 0.2 x SSC-0.1% SDS at 65°C. The blots then were exposed at -80°C to Fuji X-ray film sandwiched between two intensifying screen.

RNase protection assays

For detection of the antisense RNA in the transfected cells, an RNase protection assay was employed. Radiolabeled sense RNA probes from Cx43 cDNA containing plasmids were synthesized *in vitro*. The sense RNA synthesis was predicted to have 539 nucleotide sequences from the start of the cDNA. This contained the 313 nucleotides-long stretch of the 1.6 kb antisense RNA.

Synthesis and purification of RNA probes

Sense strand RNA probes (riboprobes) were synthesized from *SacI* digested T7/T3 291 plasmid (Fig. 3.1) by *in vitro* transcription. T3 promoter of this digested plasmid was used for sense riboprobe synthesis. *In vitro* transcription was done in a mixture containing 2 µg DNA, 4 µl [^{32}P]-CTP (800 Ci/mmol), 3 µl NTP mix, 3 µl 5x transcription buffer, 0.9 µl 1M DTT, 0.5 µl RNasin (RNase inhibitor, Gibco-BRL), and 1.5 µl T3 RNA polymerase at 37°C for 1 hour 30 minutes. After transcription, DNA template was digested by adding 1 µl (10 units) of RNase free-DNaseI (Pharmacia, USA) to the reaction mixture and incubating for additional 15 minutes at 37°C. Typically, high specific activity probes (approximately 10^9 cpm/µg) were synthesized.

The *in vitro* transcribed probes were separated on a 5% polyacrylamide-8M urea gel. The full-length RNA bands were identified by autoradiography, excised from the gel with a scalpel, and eluted overnight at 4°C in elution buffer (0.5 M NH_4OH, 1 mM EDTA, and 0.2% SDS).

Hybridization and RNase protection

Different RNA samples were mixed with radiolabelled and purified riboprobes (at least 50,000 cpm equivalent), precipitated with NH_4OH and ethanol, and then redissolved in 20 ml hybridization buffer (80% formamide, 100 mM sodium citrate, pH 6.4, 300 mM NaOAc, pH 6.4, and 1 mM EDTA). After heating the samples in a boiling water-bath, hybridization was done overnight at 45°C. RNase protection was done by adding 1:100 dilution of RNase A and RNase T1 mixture in RNase digestion buffer at 37°C for 30 minutes. Protected RNA samples were then subjected to electrophoresis on 5% acrylamide-8 M urea gel, and signals were detected by autoradiography on Fuji-RX x-ray film.

Chapter 4

MORPHOLOGY AND CHARACTERISTICS OF THE CELLS

9L Cells

The 9L cells were originally derived from a primary brain tumor that was induced by repeated serial intravenous injections of methylnitrosourea in inbred CD Fischer (cesarean-derived Fischer) rats. A tumor designated T9 or L9 was originally identified as an anaplastic astrocytoma (Benda *et al.*, 1971; Schmidek *et al.*, 1971) and the cell line was established. This cell line has been extensively used in many treatment-directed studies and is considered a model system for brain tumor therapy (Weizsaecker *et al.*, 1981; Tapscott *et al.*, 1994). Although initially this cell line was identified as astrocytoma, later, after passage through animals, it exhibited morphological differences from the original tumor line and was reclassified as a glioblastoma multiforme and sarcoma, or gliosarcoma (Weizsaecker *et al.*, 1981; Cravioto, 1986).

Most 9L cells are regular in size and shape, and display a polygonal outline and several elongated cytoplasmic processes (Fig. 4.1 A). A few multinucleate giant cells are also present in the cultures. The cytoplasm of the 9L cells is granular, and contains numerous small vacuoles. Tumor cell nuclei are of round to oval outline with one to three nucleoli. Dimensions of 9L and C6 cells are given in the Table 4.1. Average dimensions of cell bodies are 49.3 µm (length) and 22.7 µm (width), and the mean length of the processes is 48.8 µm. The mean diameter of 9L nuclei is 17.9 µm.

9L cells produce S-100 protein, glial fibrillary acidic protein (GFAP) at the periphery, and abundant amounts of collagen and reticulin fibers, when injected intracerebrally (Cravioto, 1986), and small amounts of collagen *in vitro* (Ghahary *et al.*, 1992). Though these cells do not express *in vitro* GFAP, a cytoplasmic marker for astrocytes, its expression can be induced by mechanical trauma to 9L cells grown on coverslips (scrape-wound model system).

The 9L cell line is a good model for malignant human gliomas because it presents many characteristics regarding the action of therapeutic agents and the response of the tumor that cannot be obtained using other single systems (Weizsacker *et al.*, 1981; Tapscott *et al.*, 1994).

C6 cells

The rat glioma cell line C6 is also a nitrosourea-induced cell line (Benda *et al.*, 1968) from outbred Wistar rat. This cell line is widely used to study the neurobiology of brain tumors (Cravioto, 1986). The cells show morphological features of an astrocytoma.

Most C6 cells are bipolar fusiform with elongated, regular long processes (Fig.4.1 B). There are also small number of flat polygonal cells with short stubby processes. The cytoplasm has a ground glass appearance and is without distinctive features. Tumor cell nuclei are oval with one or two small nucleoli. Average dimensions of cell bodies in length and width are 34.4 μm and 13.1 μm, respectively. The mean length of cell processes is 41.6 μm; the mean diameter of C6 nuclei is 12.3 μm.

This cell line does not synthesize appreciable amounts of GFAP, but the cells can (like 9L cells) be induced to express GFAP in this instance by treatment with dibutyryl cyclic AMP (Raju *et al.*, 1980; Backhovens *et al.*, 1987).

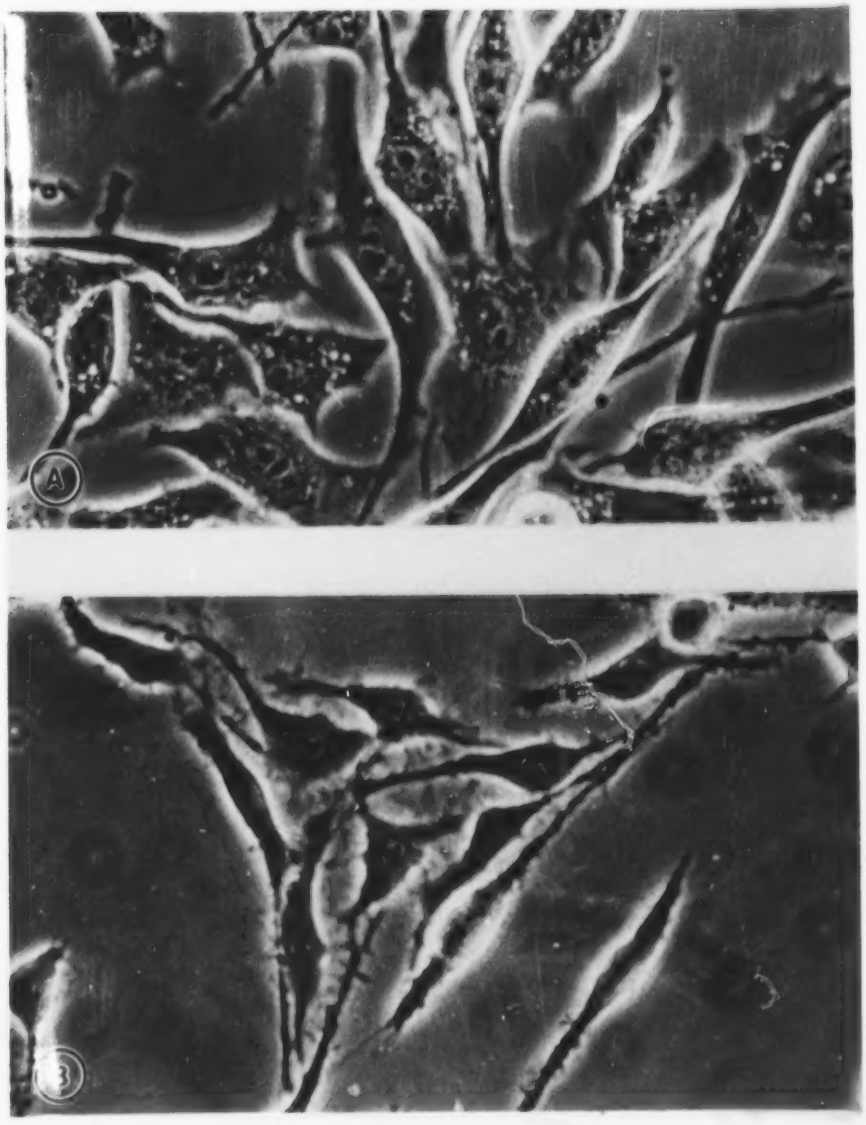
Growth rates of 9L and C6 cells

Growth curves for 9L and C6 cells were plotted as described by Freshney (1994), and the population doubling time for 9L and C6 cells in culture was determined (Fig. 4.2). The doubling time for C6 cells in the log growth phase was calculated to be about 17 hours, whereas that for 9L cells was about 18 hours. By calculating the specific growth rate for both 9L and C6 cells, using the natural logarithm of the cell numbers, the population doubling time was calculated to be 16.12 hours for C6 cells and 18.24 hours for 9L cells. In another analysis, the cell numbers were analyzed using SPSS for Window software and after plotting the logarithm of cell numbers on the ordinate and days on the abscissa, regression lines were obtained. The slope of the lines were used as the growth rates of the cells. Calculations of the population doubling times for 9L and C6 cells were done using this equation. Using these three different analyses for determining the cell

Table.4.1. Dimensions of the 9L and C6 cell-bodies, processes and their respective nuclei were measured from the phase-contrast micrographs of the cells. Values are shown with +/- one standard deviation; n is the number of measurements taken from different cells.

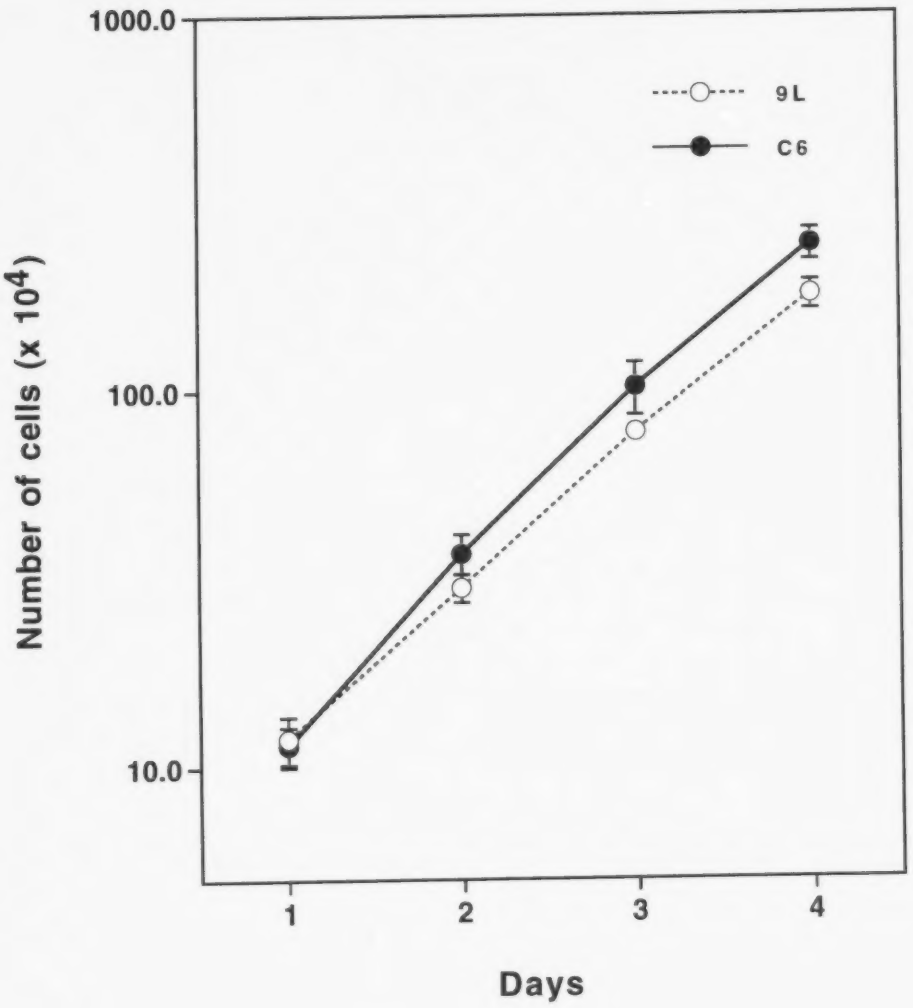
Cell	***Length (μm)***	***Width (μm)***	***Length of Processes (μm)***	***Size of Nuclei (μm)***
9L	49.3 +/- 1.6 (n= 31)	22.7 +/- 1.7 (n= 31)	48.8 +/- 4.8 (n= 31)	17.9 +/- 0.4 (n= 31)
C6	34.4 +/- 1.3 (n= 35)	13.1 +/- 0.5 (n= 35)	41.6 +/- 3.4 (n= 35)	12.3 +/- 0.3 (n= 27)

Fig. 4.1. Phase-contrast micrographs of 9L (A) and C6 (B) cells. Cells were grown on glass-coverslips and photographed in PBS as mounting medium. The 9L cells (A) show larger cells with one or more rounded or oval nuclei. The C6 cells (B) are smaller with rounded or oval nuclei. Magnifications= 570x.


A
B

growth rates of the two cell lines, similar results were obtained. Thus, the growth rates for both of the cell lines in cultures were similar.

Fig. 4.2. Comparison of growth of 9L (broken line) and C6 (solid line) cells in culture. The cells were grown in triplicates and cells from three flasks were counted daily. Graph was plotted on a semi-logarithmic scale with the Y-axis being logarithmic. Vertical bars represent one standard deviations (n= 3).


1000.0
100.0
10.0
Number of cells (x 10^4)
9L
C6
1
2
3
4
Days

Chapter 5

COMPARISON OF GAP JUNCTIONAL CHARACTERISTICS OF 9L AND C6 GLIOMA CELLS

Immunofluorescence labeling of gap junctions

9L and C6 cells were analyzed by indirect immunofluorescence microscopy to determine the distribution of gap junction connexins. Cells grown on glass coverslips were labeled with affinity purified antibodies that were produced against a synthetic peptide corresponding to a carboxyl-terminal sequence of the Cx43 protein (Risek *et al.*, 1992). A punctate pattern of immunofluorescence was detected along the course of cell membranes. This labeling at the periphery of the cells represents the gap junction plaques (Hertzberg, 1985). 9L cells (Fig. 5.1 A) displayed stronger, more abundant staining at the cell periphery, and also cytoplasmic staining in the perinuclear area, which presumably corresponded to newly synthesized Cx43. Most of the C6 cells were not labelled by α_1s antibodies. However, a few C6 cells showed labeling with antibodies to Cx43 (Fig. 5.1 C); these cells were rare. The labeling in C6 cells was less apparent than in 9L cells and only a few fluorescent spots were detectable along the cell periphery.

These results indicated that, although both 9L and C6 cells form Cx43 gap junctions, the extent of gap junction formation and the level of Cx43 expression was considerably higher in 9L cells than in C6 cells.

Gap junctional ultrastructure in 9L and C6 cells

Intercellular contacts and gap junctions in the 9L and C6 cells were analyzed by thin section electron microscopy. 9L cells exhibited cell membrane contacts that included gap junctions. Impregnation of the intercellular space with lanthanum delineated the gap junctions in the 9L (Fig. 5.2) and C6 cells (Fig. 5.3). Gap junctions observed between 9L cells were larger, more frequent, and better-defined (Fig. 5.2 A, B, C, D, E, and F) in comparison to the gap junctions between C6 cells (Fig. 5.3 A, B, C, D, E, and F). Plasma membranes of neighboring C6 cells were seen to run together and come closer to form

Fig. 5.1. Immunofluorescence micrographs of 9L (A and B) and C6 cells (C and D) labeled with α_1s antibodies to connexin43. The 9L cells contain numerous gap junctions along the cell-to-cell contact area and also show strong intracellular cytoplasmic staining (A). Fewer C6 cells are labeled with the α_1s antibodies (C), and the labeling is mostly limited to the periphery of these cells. Pre immune serum was used as negative controls for 9L (B) and C6 (D). Magnifications= 630x.

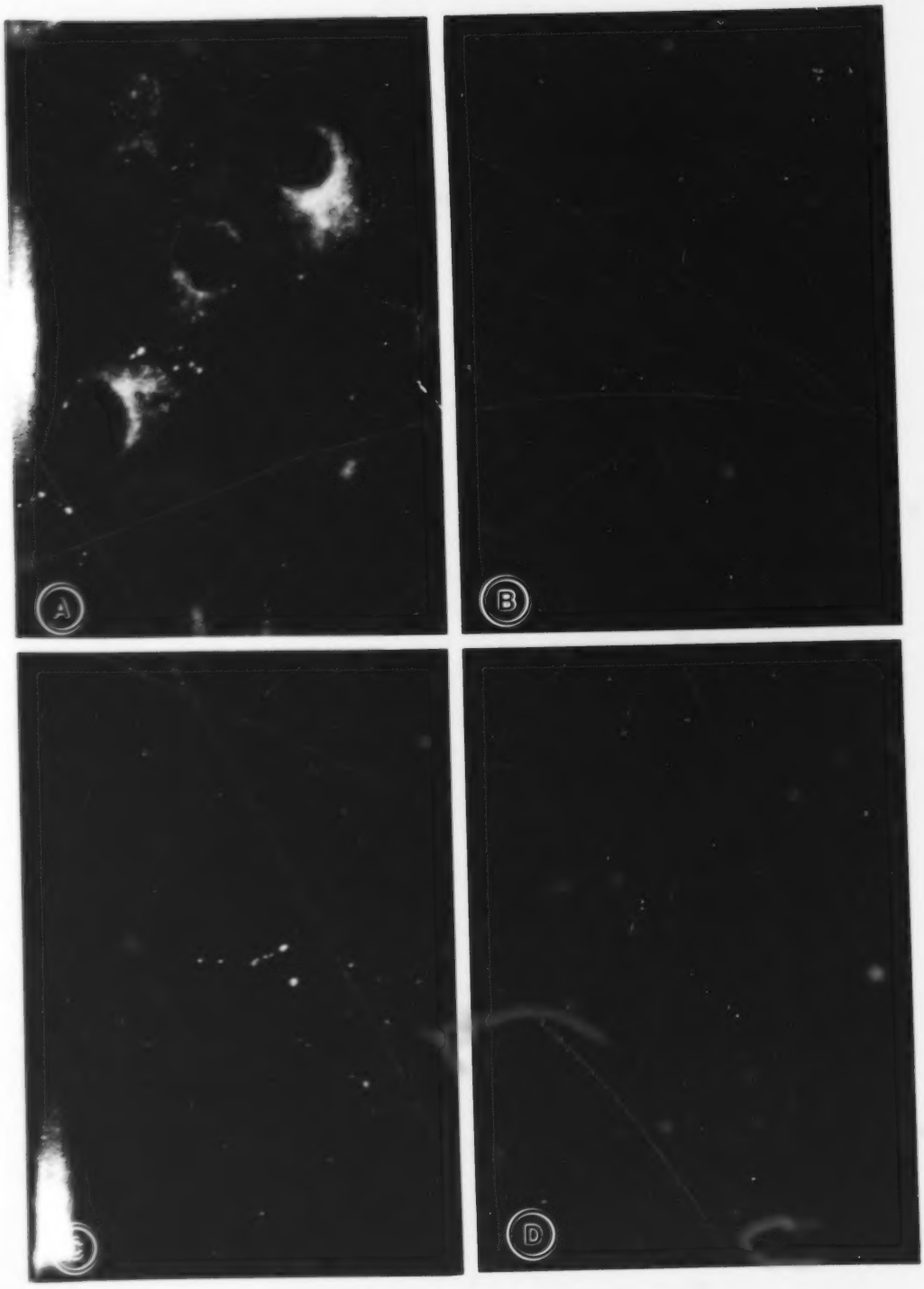
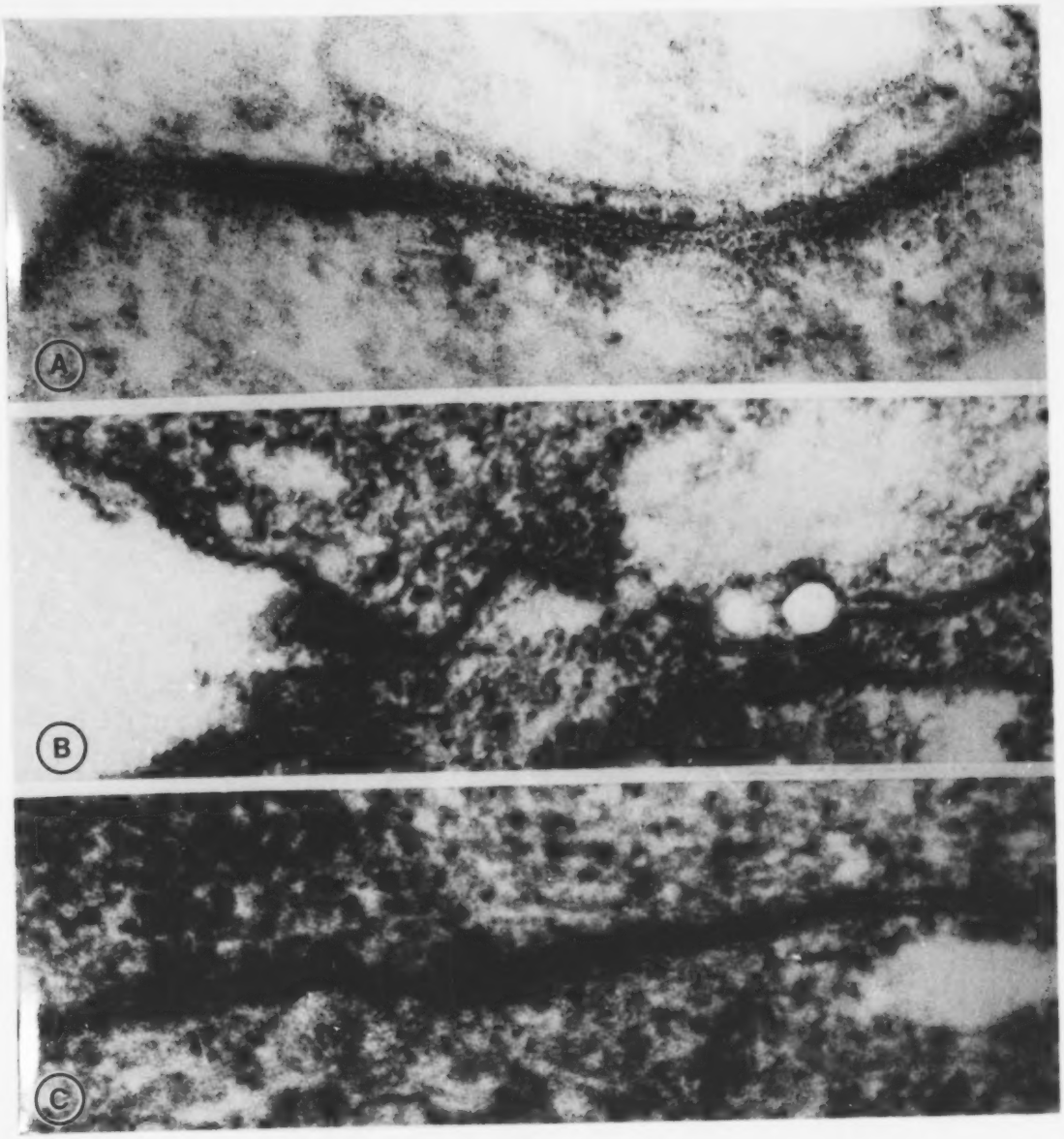

A
B
C
D

Fig. 5.2. Electron micrographs showing gap junctions between 9L cells (A, B, C, D, E, and F). The 9L cells were impregnated with lanthanum hydroxide colloidal solution during fixation. These cells formed large gap junctions which were frequent.


A
B
C

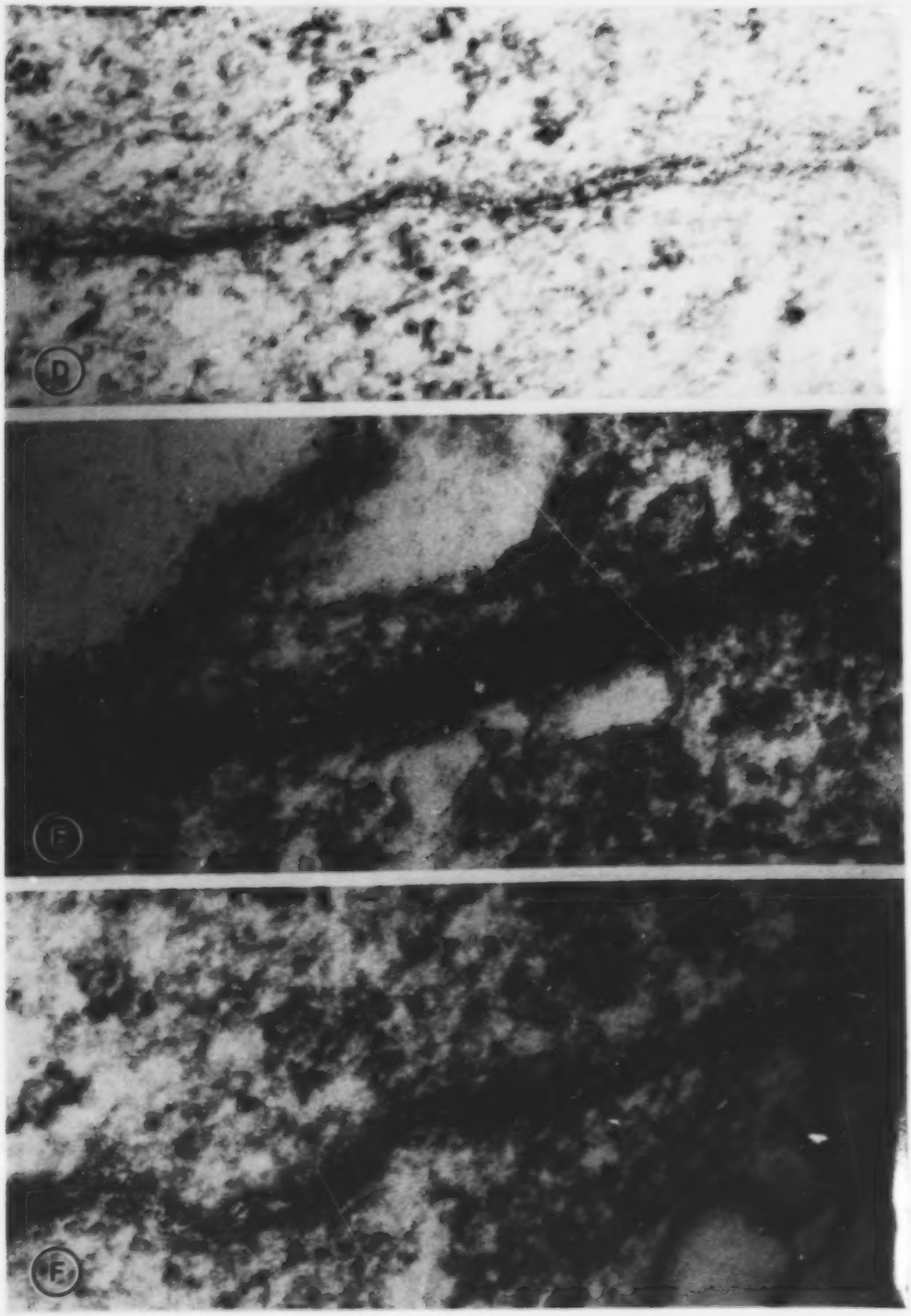
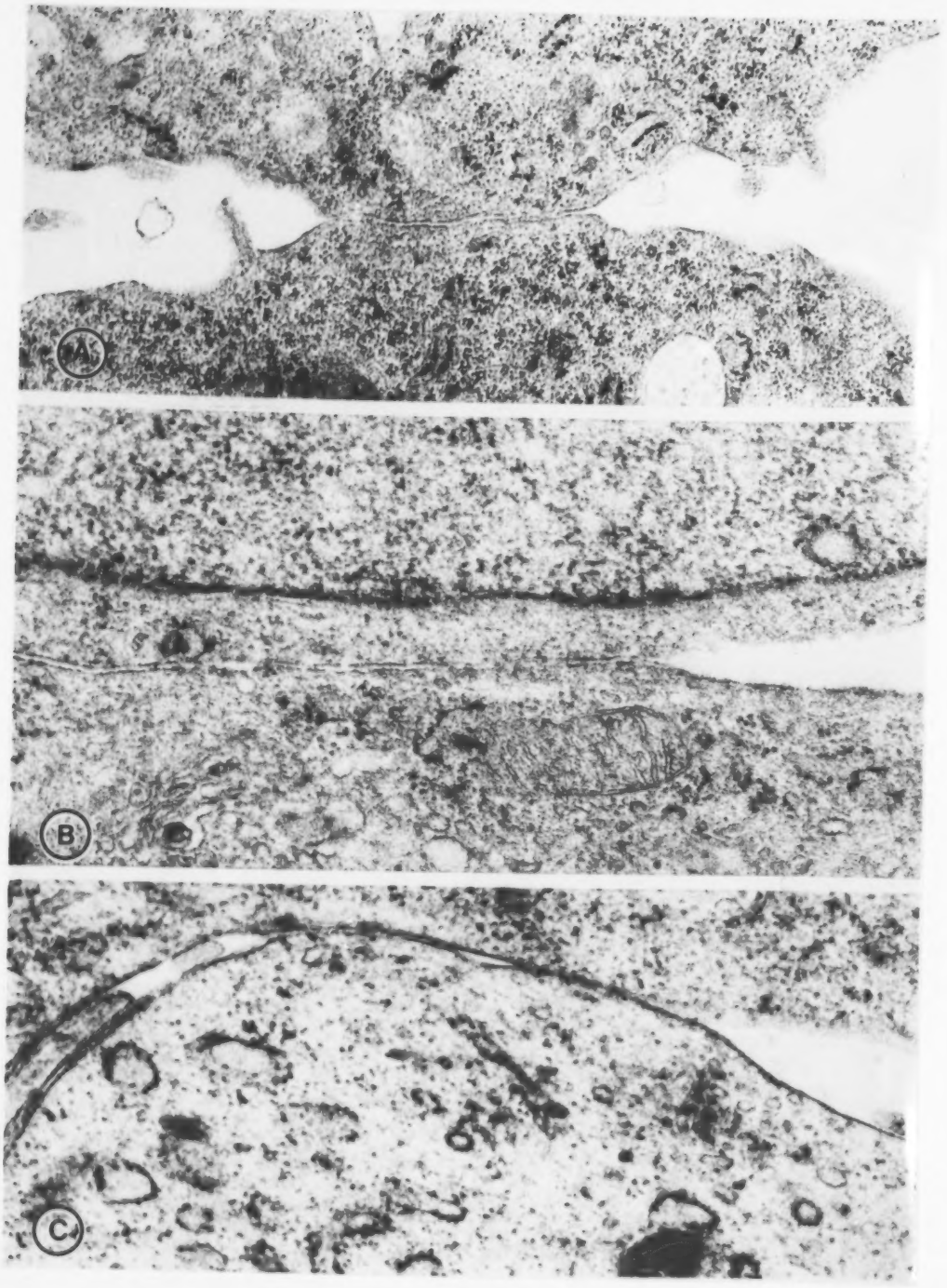

D
F
F2

Fig. 5.3. Electron micrographs showing apposing membranes of C6 cells (A, B, C, D, E, and F). The cells were impregnated with lanthanum hydroxide colloidal solution during fixation. Membranes of the cells were closely apposed (A, B, C, and F), but they formed "kissing junctions" (D and E).


A
B
C

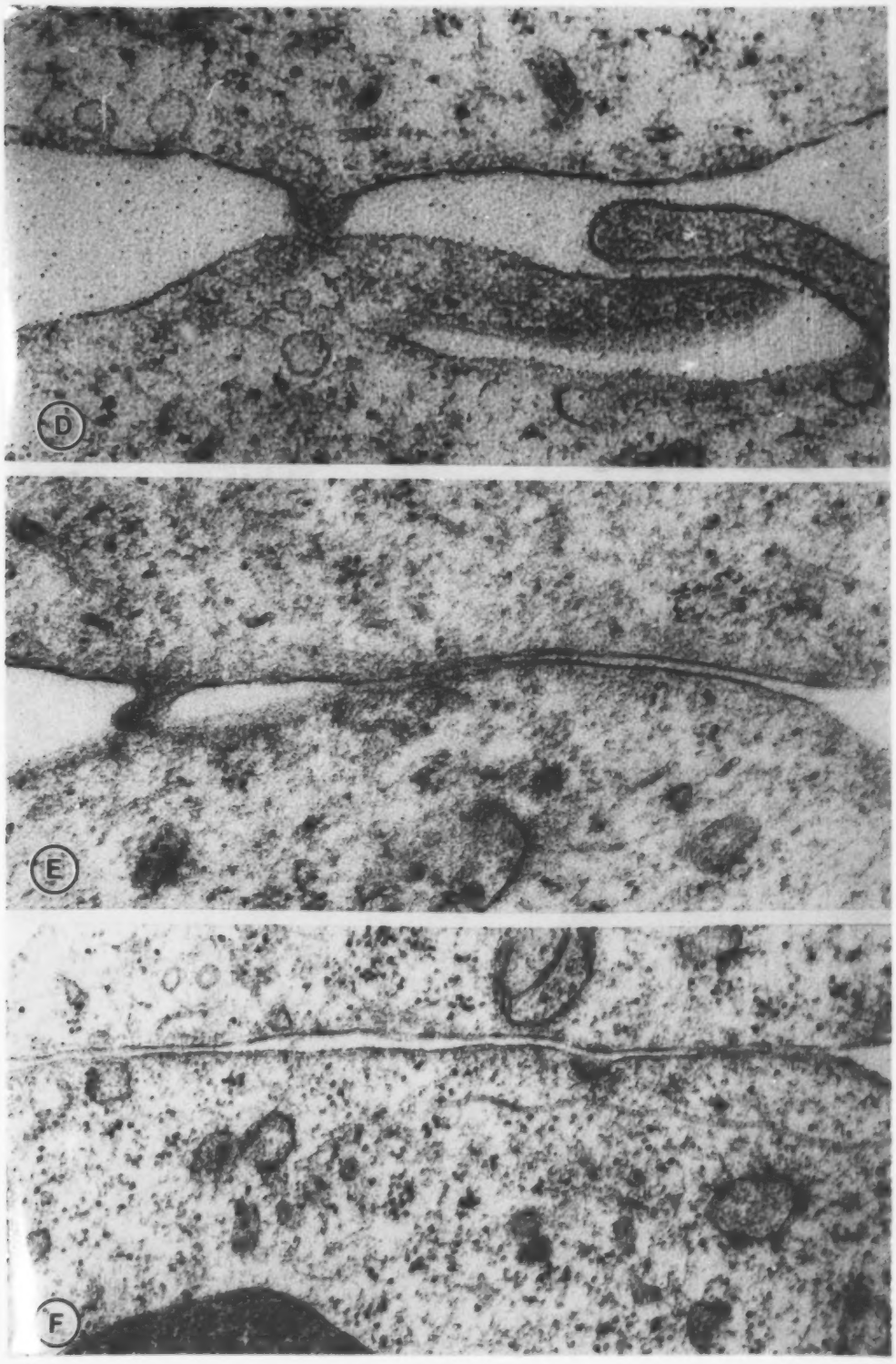
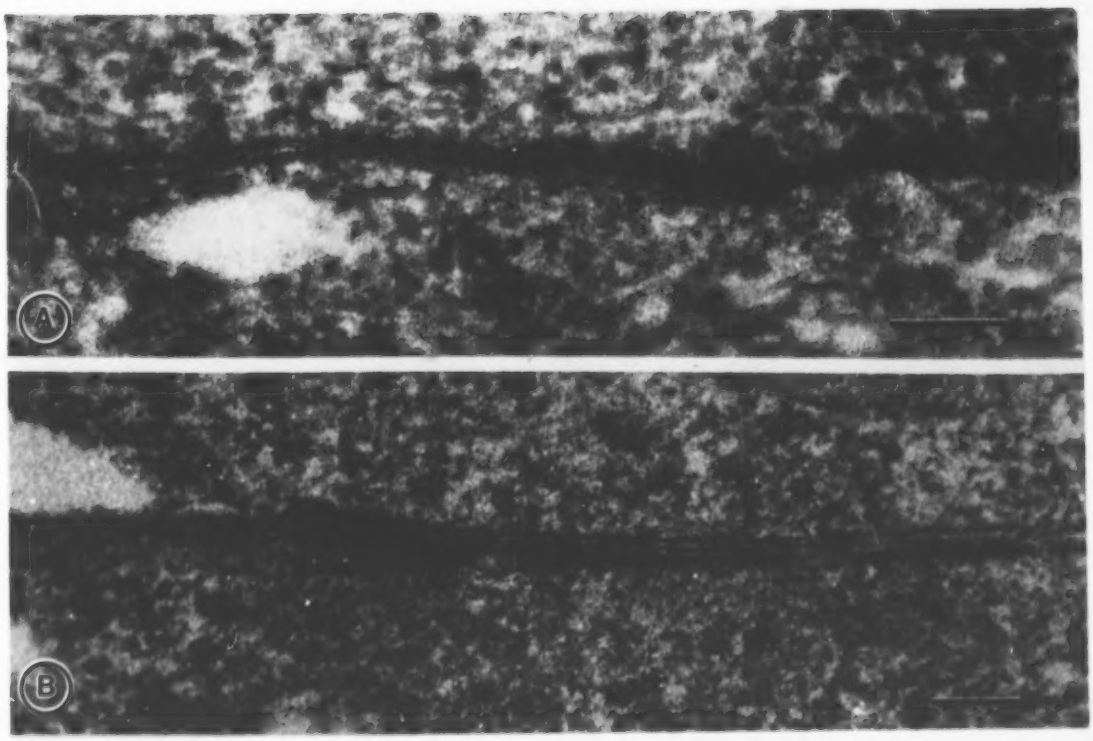

D
E
F

Fig. 5.4. Electron micrographs of lanthanum impregnated 9L (A) and C6 (B) gap junctions. The electron micrographs here show typical gap junctions between the respective cells. Scale bars = 200 nm.


A
B

'kissing junctions' (Fig. 5.3 D and E), but they seldom made large gap junctional contacts (Fig. 5.4 B).

These results indicate that both 9L and C6 cells formed gap junctions between adjacent cells. However, the gap junctions between 9L cells were larger in size and were more readily observed than in C6.

Dye coupling between cells

The presence of gap junctions and their molecular components is not evidence of intercellular communication because the opening and closing of gap junctional channels are regulated by intracellular and extracellular conditions (see Chapter 2). Therefore, dye transfer between cells was examined to determine the extent of gap junctional communication between 9L and C6 cells, respectively. With the scrape loading/dye transfer method, in three different experiments, glass coverslips were scraped in duplicate and the transfer of Lucifer Yellow CH dye to adjacent cell layers was monitored. In three such experiments, 9L cells consistently showed spread of the dye to several layers of neighboring cells (Fig. 5.5 A), whereas in C6 cells grown on coverslips the dye seldom diffused to one or two layers of cells (Fig. 5.5 B). The cells distal to the scrapes did not show dye uptake, confirming that the dye did not diffuse through the plasma membrane of the cells, and that the diffusion of dye to neighboring cells was through intercellular channels. Thus, in accordance with the results obtained from ultrastructural and immunofluorescence labeling, the 9L cells exhibited more extensive gap junctional communication than did the C6 cells.

Immunoblotting for connexin43

The expression of connexins in 9L and C6 cells was examined by immunoblotting. However, it is possible that cells that synthesize connexin may fail to form functional gap junctions, because of defects in post-translational events (Musil and Goodenough, 1990). The abundance of connexin43, therefore, was checked using three different sample preparations. The samples were prepared from (a) an alkali insoluble fraction (which enriches gap junctional plaques from cell membranes) from equal numbers of cells of each cell line (Fig. 5.6), (b) equal amounts of total protein in crude cell homogenates (Fig. 5.7, lanes B and C), and (c) equal amounts of protein from the alkali insoluble fractions of the cells (Fig. 5.7, lanes D and E).

Fig. 5.5. Fluorescence photomicrographs of scrape loading/ dye transfer experiments in 9L and C6 cells. In the 9L cells (A) the intercellular diffusion of fluorescent dye Lucifer Yellow CH is greater than in C6 cells (B) under similar conditions. Asterisks show the line of scrape. Magnification= 400x.

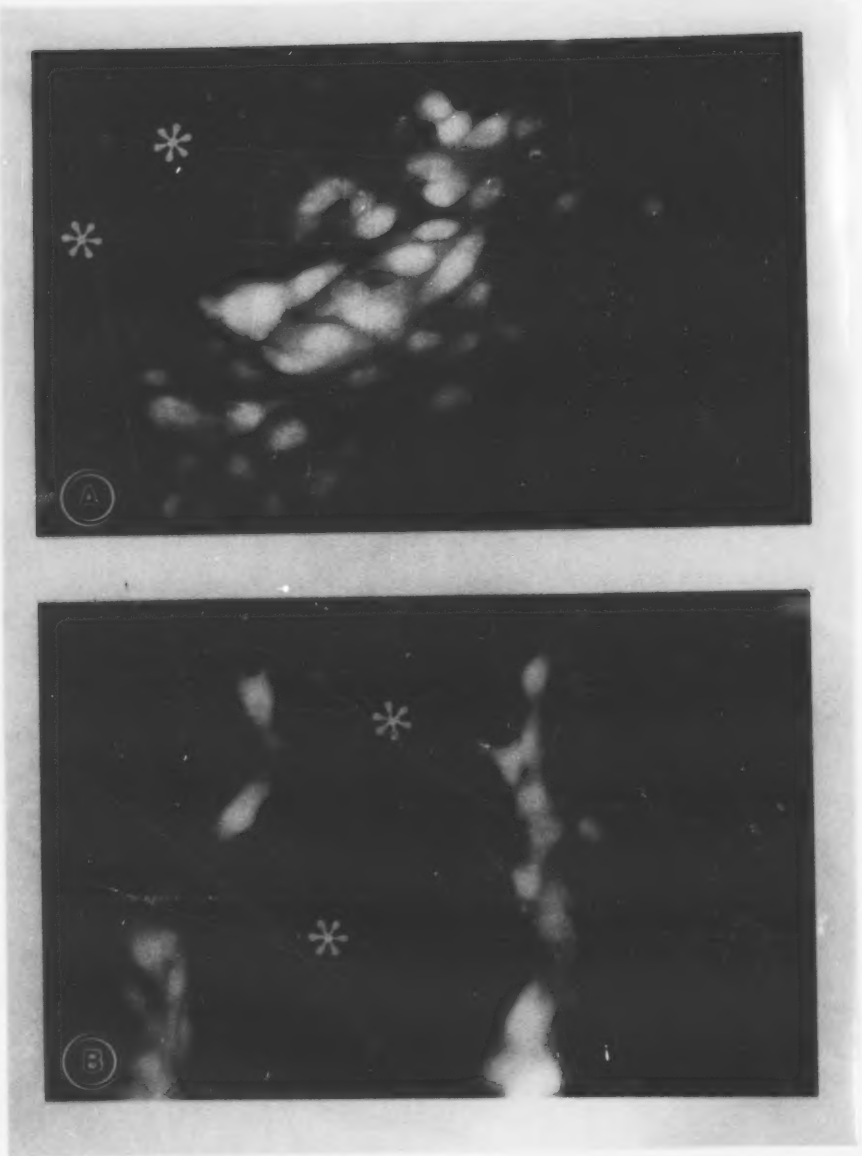

A
B

Fig. 5.6. Immunoblotting of 9L and C6 cell alkali insoluble proteins for connexin43. Equal numbers of 9L (lane A) and C6 (lane B) cells were used to prepare alkali insoluble protein samples. Homogenates and alkali-insoluble samples were separated by SDS-PAGE, transferred to a nitrocellulose membrane, and probed with α_1s antibodies. The antibodies recognize Cx43 bands of 42- and 44 kD mobility in the 9L lane. These connexin bands were not detected in the C6 samples, although a larger protein band is recognized in both the samples, which is probably due to aggregation of Cx43 with other proteins (Hertzberg, 1985). Numbers on the left side indicate the size of standard molecular weight markers (BioRad, USA) in kilodaltons.

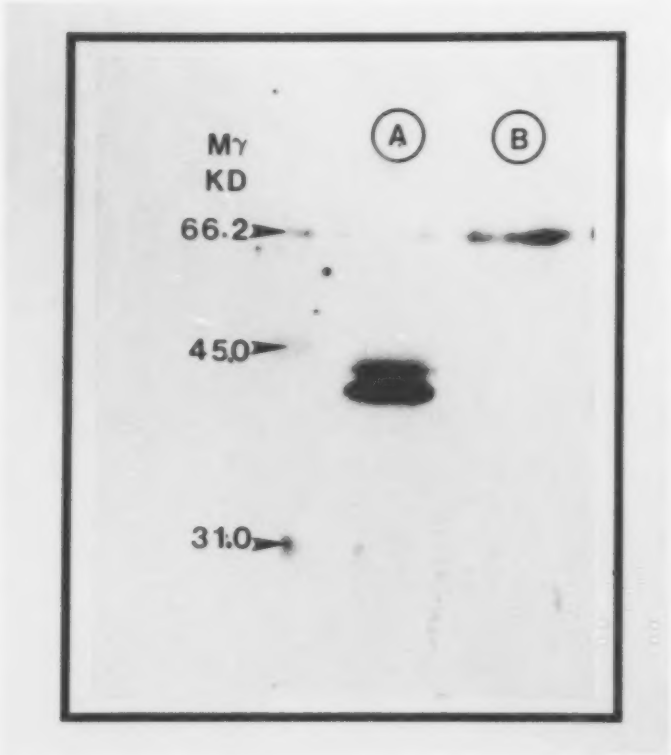
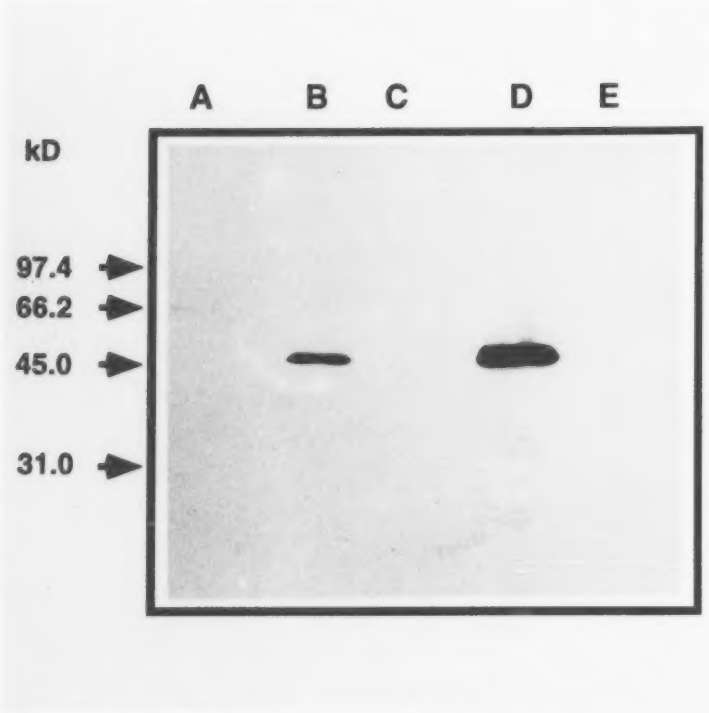

Mγ
KD
66.2
45.0
31.0
A
B

Fig. 5.7. Immunoblotting of whole cell homogenates and alkali insoluble proteins of 9L and C6 cells. Equal amounts of total cellular proteins from 9L (lane B) and C6 (lane C), and from alkali insoluble 9L (lane D) and C6 (lane E) cell samples were separated by SDS-PAGE, transferred to a nitrocellulose membrane, and probed with α1s antibodies. The antibodies recognize strong connexin43 protein bands in the 9L lanes containing cell homogenate (lane B) and alkali insoluble fraction (lane D). The connexin band was not detected in the C6 cell homogenate sample (lane C); in the alkali insoluble fraction of C6 cells, however, a faint connexin43 band is seen. Lane A contained the molecular size markers. Numbers on the left side indicate the size of molecular weight markers in kilodaltons.


A
B
C
D
E
kD
97.4
66.2
45.0
31.0

In all the samples from 9L cells (Fig. 5.6, lane A, and Fig. 5.7, lanes B and D), connexin43 protein bands were readily detectable on the immunoblots with α_1s antibodies. However, corresponding lanes of C6 samples equally loaded with total protein from crude cell homogenate (Fig. 5.7, lane C), did not show detectable connexin43. With the alkali insoluble samples of cells which contained equal amounts of protein, a faint connexin43 band was detected in the C6 lane (Fig. 5.7, lane E). Although in Fig. 5.6 the C6 lane does not show a band at 43 kD, a higher band is visible which probably arises from the aggregation of connexin protein.

Cx43 bands migrated as 42 kD and 44 kD proteins in size in the 9L cell samples. The higher molecular weight band probably represented the phosphorylated form of the 42 kD protein band (Brissette *et al.*, 1991). The Cx43 band, however, was not detectable in C6 cell samples.

Northern blot analysis

Expression of Cx43 and Cx26 mRNA as well as the relative amounts of Cx43 mRNA in total cellular RNA of 9L and C6 cells were compared on Northern blots using RNA probes. Approximately equal amounts of total cellular RNA from 9L and C6 cells were separated on 1.4% agarose-formaldehyde gel and blotted on nitrocellulose membranes. Hybridization of the blots to radiolabeled probes from Cx43 cDNA followed by washing under stringent conditions (0.5 X SSC, 0.1% SDS, 65°C) yielded one band of Cx43 mRNA on the autoradiograms (Fig. 5.8). Both the lanes containing 9L (Fig. 5.8, lane A) and C6 (Fig. 5.8, lane B) cellular RNA showed the presence of a Cx43 mRNA band. The size of this RNA was determined to be approximately 3.0 kb based on the RNA molecular size markers run in parallel on the same gel. As a loading control, β-actin probe was used. Reprobing the stripped blot with a radiolabeled probe for mouse β-actin mRNA showed a single band in both 9L (Fig. 5.8 lane C) and C6 (Fig. 5.8, lane D) at about 2.0 kb position. Band intensities of Cx43 and β–actin signals were determined by optical densitometry of the autoradiograms. Normalization of Cx43 RNA band intensities with their respective β-actin band intensities showed that the level of mRNA in 9L cells was about six-fold higher than that in C6 cells (Fig. 5.9).

When Northern blots of 9L and C6 RNAs were hybridized with a radiolabeled probe from a Cx26 cDNA (Fig. 5.10), both 9L (lane B) and C6 (lane C) did not show any

Figure.5.8. Expression of Cx43 mRNA in 9L and C6 cells. Northern blot analyses were done to detect mRNAs of Cx43 and β-actin in 9L and C6 total RNA samples. An approximately 3 kb size Cx43 RNA band was detected in both the samples. The 9L total RNA sample (lane A) shows higher signal intensity than C6 (lane B) for Cx43. Hybridization of the same RNA blot with a RNA probe of mouse β-actin (loading control) showed a similar amount of total RNA loading in both 9L (lane C) and C6 (lane D) RNA samples. The RNA size markers were run in parallel, and their positions are shown on the left side.

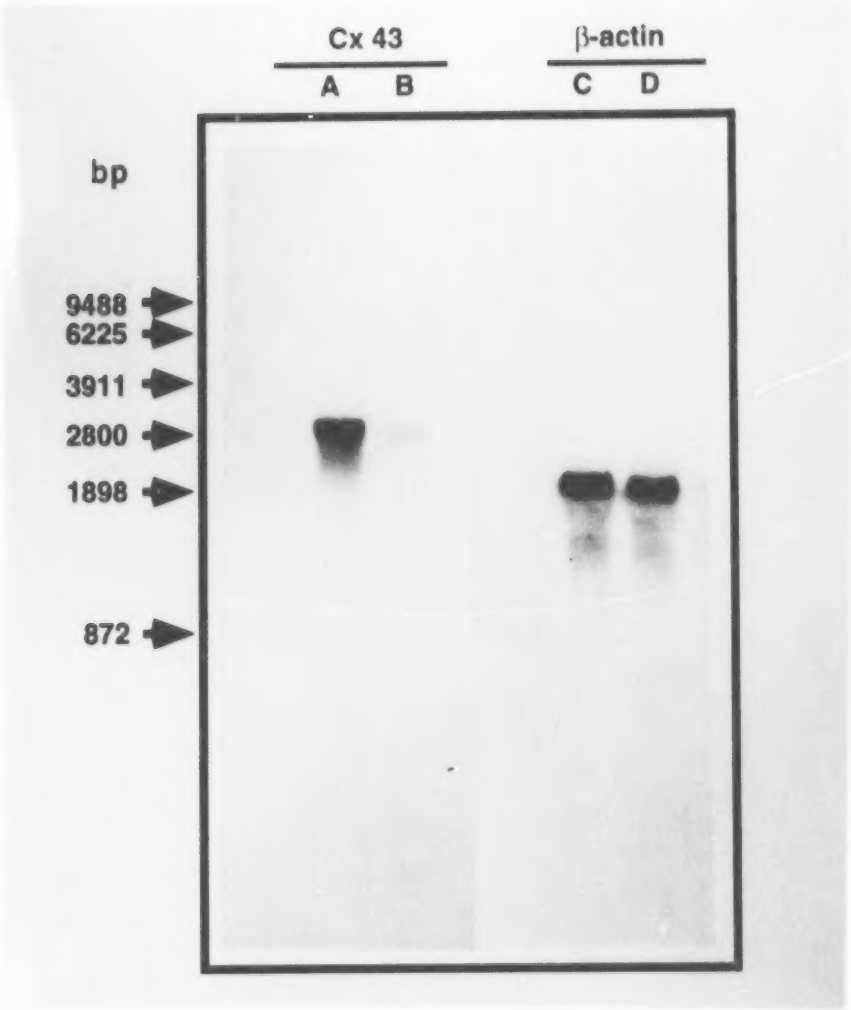

Cx 43
β-actin
A
B
C
D
bp
9488
6225
3911
2800
1898
872

Fig. 5.9. Relative expression of Cx43 mRNA in 9L and C6 cells. Signal intensity ratios of Cx43 and β-actin for 9L and C6 Northern blots are shown here for comparison. Optical density of Cx43 and β-actin RNA bands on the autoradiographs were measured by optical densitometry and their ratio were determined. The 9L cells show more than a six-fold greater expression of Cx43 than in C6 cells.

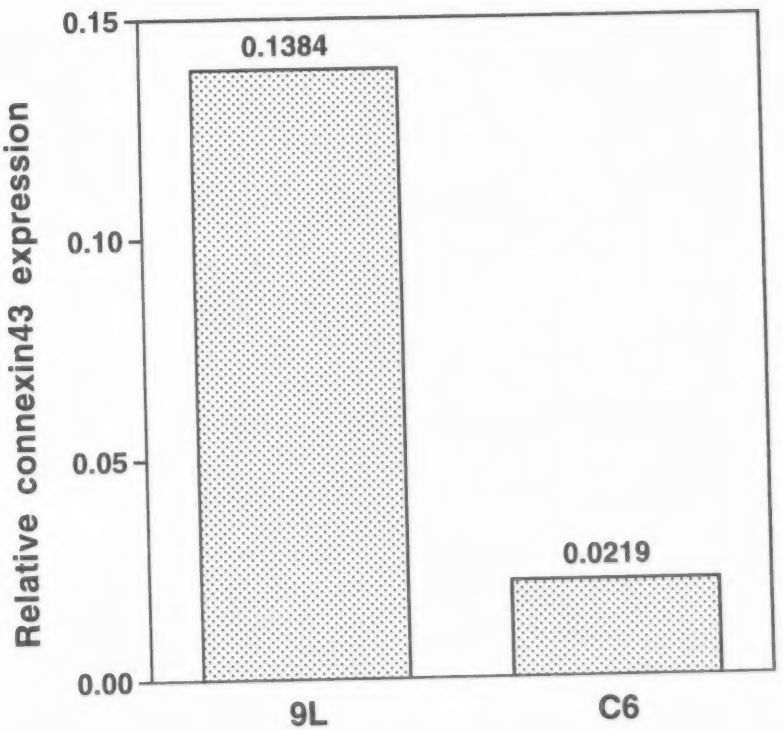
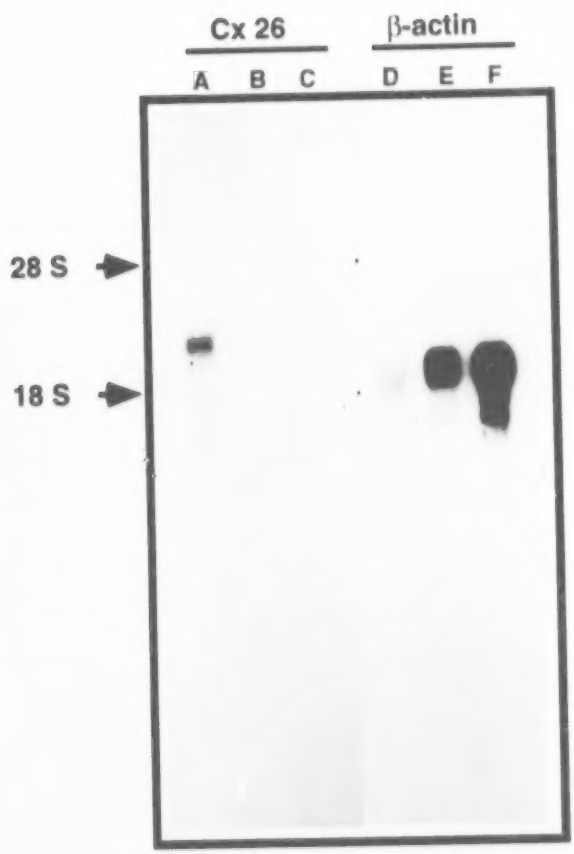

Relative connexin43 expression
0.15
0.10
0.05
0.00
0.1384
0.0219
9L
C6

Fig. 5.10. Detection of Cx26 mRNA in mouse liver, 9L cells and C6 cells. Northern blot analyses to detect Cx26 mRNA in 9L (lane B) and C6 (lane C) total RNA samples. Mouse poly A^+ RNA (lane A) is a positive control. The 9L and C6 RNA samples (lanes B and C, respectively) do not show signal for Cx26, whereas mouse liver poly A^+ RNA (lane A) does. Hybridization of the same RNA blot with a riboprobe for mouse β-actin (loading control) showed that relative levels of RNA in 9L (lane E) and C6 (lane F) samples were higher than mouse liver sample (lane D). Positions of 18 S and 28 S ribosomal RNA bands is shown on the left side.


Cx 26
β-actin
A B C D E F
28 S
18 S

bands, whereas a mouse liver polyA$^+$ RNA sample in the adjacent lane (lane A), which was used as a positive control, showed a distinct mRNA band. Probing the same blot for β-actin showed that the 9L and C6 (respectively lanes E and F) had relatively more poly A$^+$ RNA than the positive control (lane D). This indicated that both 9L and C6 cells do not synthesize Cx26 RNA, or that the level of Cx26 expression in these cells is not detectable.

Taken together, these results show that 9L and C6 rat glioma cells are different with regards to their gap junctional characteristics. Both cells express one type of connexin, namely connexin43. The relative levels of connexin43 expression in these two cell lines, as judged by immunofluorescence labeling, immunoblotting, and Northern blotting, are different, with 9L cells expressing more connexin43 RNA and protein than C6 cells. These differences are reflected in their respective gap junctional ultrastructures and dye transfer abilities.

Chapter 6

TRANSFECTION OF 9L GLIOMA CELLS AND INHIBITION OF CONNEXIN43 SYNTHESIS

Transfection of 9L cells

The 9L cells were co-transfected with pNuTBGHE and pSFFV.neo plasmids. The pNuT BGHE plasmid contains approximately 1.6 kb long *HindIII* fragment of Cx43 cDNA which is ligated downstream to a mouse metallothionein (MT) promoter in reverse orientation, to synthesize antisense RNA, and its transcription is under the MT promoter's control. The co-transfected plasmid pSFFV.neo (Fuhlbrigge *et al.*, 1988) contains the selectable marker gene for neomycin resistance, and is used for selecting transfected eukaryotic cells for resistance to geneticin (G418, Gibco-BRL). Transfection was performed using Lipofectin reagent (Gibco-BRL) according to the manufacturer's protocol as detailed in the materials and methods chapter.

After transfection, cells were subjected to selection by growing in G418 supplemented medium. The effective concentration of G418 for selection was determined by growing untransfected 9L cells in different concentrations of G418 in culture medium, and monitoring the number of cells after one week of growth. Figure 6.1 shows the results obtained from exposure of 9L cells to various concentrations of G418. The cells were severely affected by addition of 260 µg /ml G418 in the growth medium. Therefore, this concentration was chosen for the selection of G418-resistant transfected cells. A number of cell colonies were seen to be formed after growth for two weeks in the selection medium. Large and healthy colonies were marked and cells were isolated using cloning cylinders. From different colonies, eleven transfectant clones were obtained and aliquots of their cells were frozen. After preliminary screening for inhibition of connexin synthesis by immunofluorescence labeling, an AB5 clone was selected for further studies.

Southern blot analysis for transfected DNA

Although selection in G418 containing medium indicates the transfection of DNA, the G418 resistance marker was located on a different plasmid. Therefore, Southern blot analysis was performed to determine successful transfection of antisense connexin

Fig. 6.1. G418 (Geneticin) sensitivity of 9L cells. The 9L cells were grown in presence of increasing concentration of G418 antibiotic, and the cell counts were taken. The bar-diagram shows that at a concentration of 260 μg per ml, the cells were severely affected. Vertical bars show the standard deviation of the cell counts data.

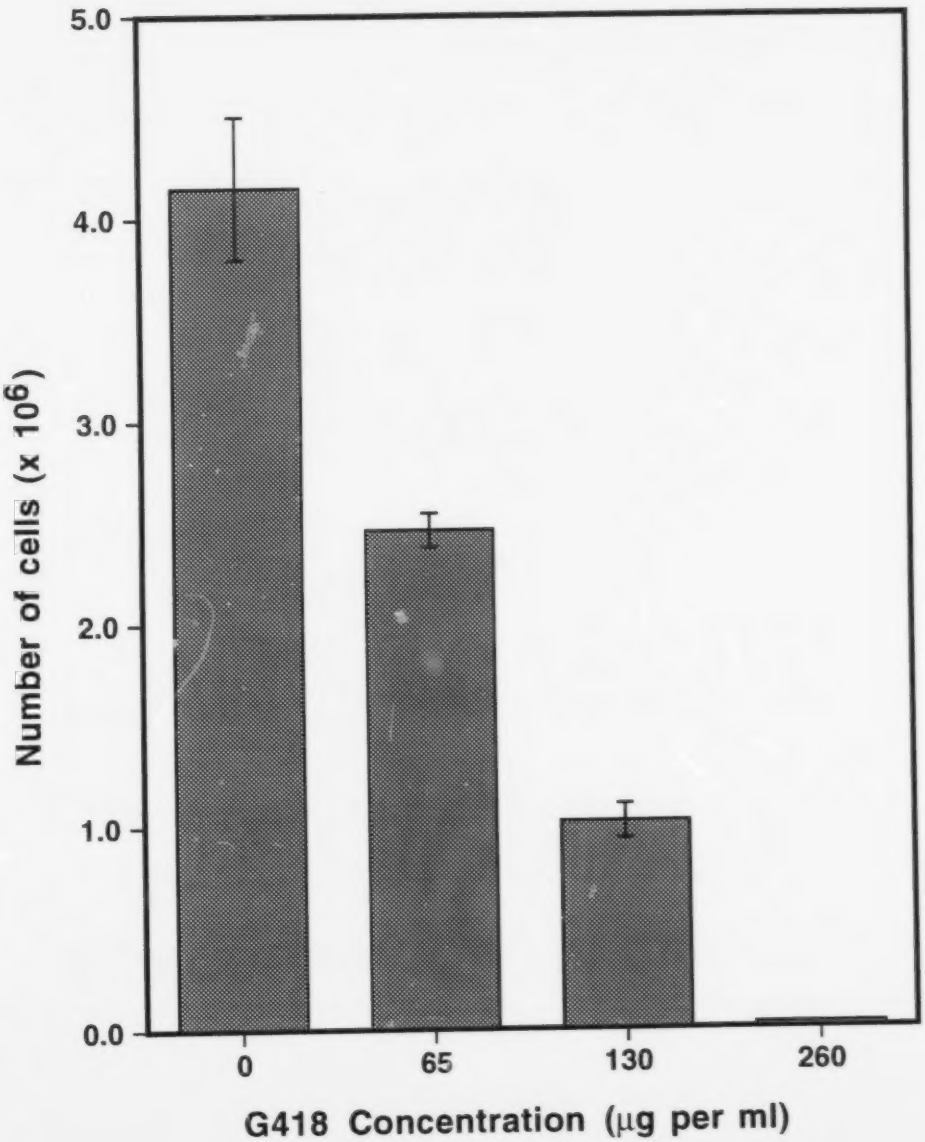
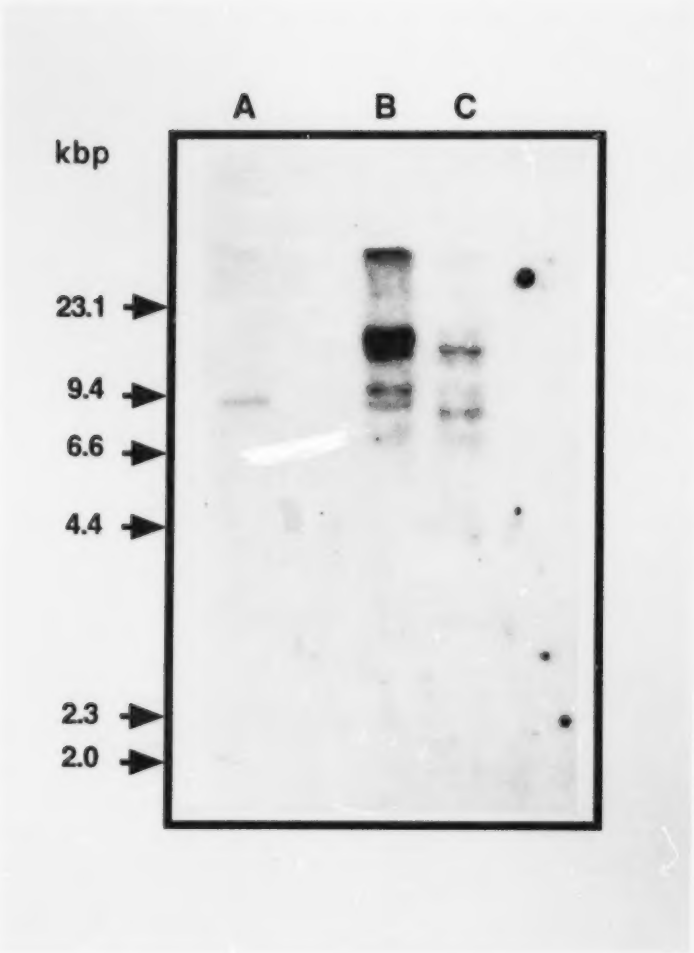

5.0
4.0
3.0
2.0
1.0
0.0
Number of cells (x 10^6)
0
65
130
260
G418 Concentration (μg per ml)

Fig. 6.2. Southern blot of untransfected 9L (lane A) and transfected AB5 (lane C), and another transfected AC4 (lane B) genomic DNA after restriction with *PstI* endonuclease. The Cx43 cDNA probe hybridized to only one band in the lane containing DNA from 9L cells (lane A), whereas lanes containing AC4 (lane B) and AB5 (lane C) DNA hybridized to addtional bands. Arrows at the left indicate the size of λ phage DNA digested with *HindIII* restriction enzyme (in kilobase-pairs).


A
B
C
kbp
23.1
9.4
6.6
4.4
2.3
2.0

plasmid. Total cellular DNA from untransfected 9L and transfected AB5 cells were isolated and blotted after overnight digestion with *PstI* endonuclease and separation by electrophoresis. An autoradiogram of the blot, after probing with a nick-translated Cx43 probe, is shown in Fig. 6.2. The lane containing the untransfected 9L DNA shows a single connexin band, whereas transfected AB5 and another transfected clone AC4 show additional bands, along with the original connexin43 DNA band.

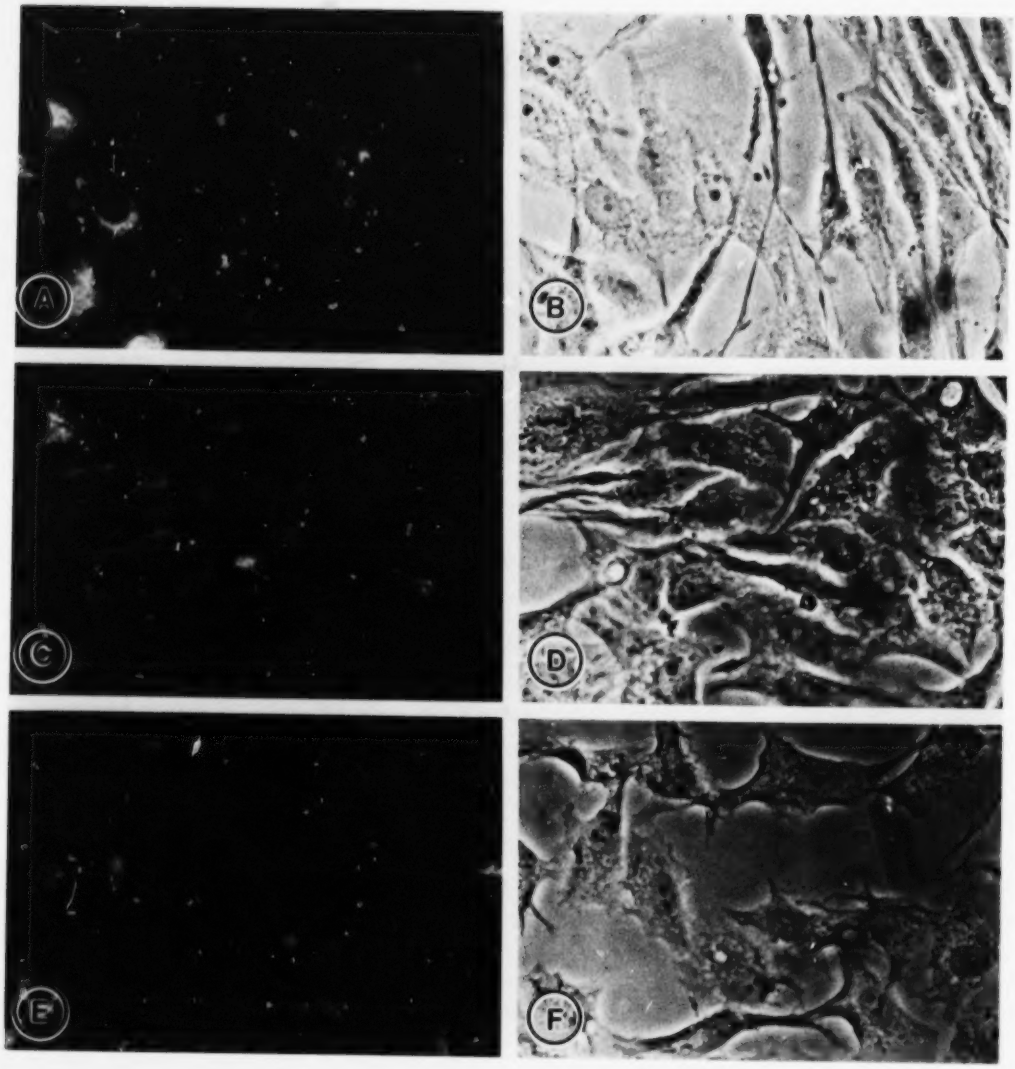
Thus, the Southern blot indicates that transfected cells have additional copies of connexin43 DNA indicating successful transfection. Several hybridizing bands indicate that multiple copies of the plasmid are present in the cells.

Loss of connexin43 gap junctions in transfected cells

Immunofluorescence labeling

Expression of antisense connexin43 RNA would result in inhibition of connexin43, thereby reducing the number of gap junctions at the intercellular contacts. To determine the inhibition of connexin43 synthesis by antisense RNA expression, transfected AB5 cells were grown on glass coverslips, and were treated with 75 μM zinc acetate. After 6, 12, 24, and 36 hours of treatment, cells were fixed and immunolabeled with α_1s antibodies. The results are shown in Fig. 6.3 and Fig. 6.4. It is obvious from the micrographs that cells from untreated petri-dishes formed gap junctions which are seen to be arranged in a typical punctate labeling pattern at the cell-cell interface (Fig 6.3 A and Fig. 6.4 A). This labeling at the cell-cell interface is seen even after 6 hours of zinc treatment (Fig. 6.3 C and Fig. 6.4 C). However, after 12 hours of zinc treatment the number of punctate spots at the cell-cell interface decreased (Fig. 6.3 E and Fig. 6.4 E). This labeling was almost completely eliminated after 24 hours of zinc treatment (Fig. 6.3 G and Fig. 6.4 G). Even 36 hours after the addition of zinc acetate, the number of gap junctions remained low (Fig. 6.3 I and Fig. 6.4 I). Pre-immune serum controls for each time point did not show labeling (panels B, D, F, H, and J in Figures 6.3 and 6.4). The untransfected 9L cells were also treated similarly and immunolabeled with α_1s antibodies. The 9L cells, however, did not show decrease in labeling of gap junctions after zinc acetate treatments (Fig. 6.6, panels A, C, E, G, and I). In another experiment, when a mixture of zinc acetate (50 μM) and cadmium chloride (2 μM) was added to the cultures to induce Cx43 antisense RNA expression, labeling with α1s antibodies was completely abolished after a 24 hour treatment with cadmium chloride (Fig. 6.5).

Fig. 6.3. Immunofluorescence labeling of AB5 cells after antisense expression by zinc treatment. Culture grown cells were treated with zinc acetate, and immunofluorescence labeling using α1s antibodies was done after 6, 12, 24, and 36 hours of the treatment. Figures A, C, E, G, and I show the labeling of, respectively, untreated, 6-, 12-, 24 - and 36 hours of treatment. Figures B, D, F, H, and J are corresponding phase-contrast micrographs. Magnification= 400x.


A
B
C
D
E
F

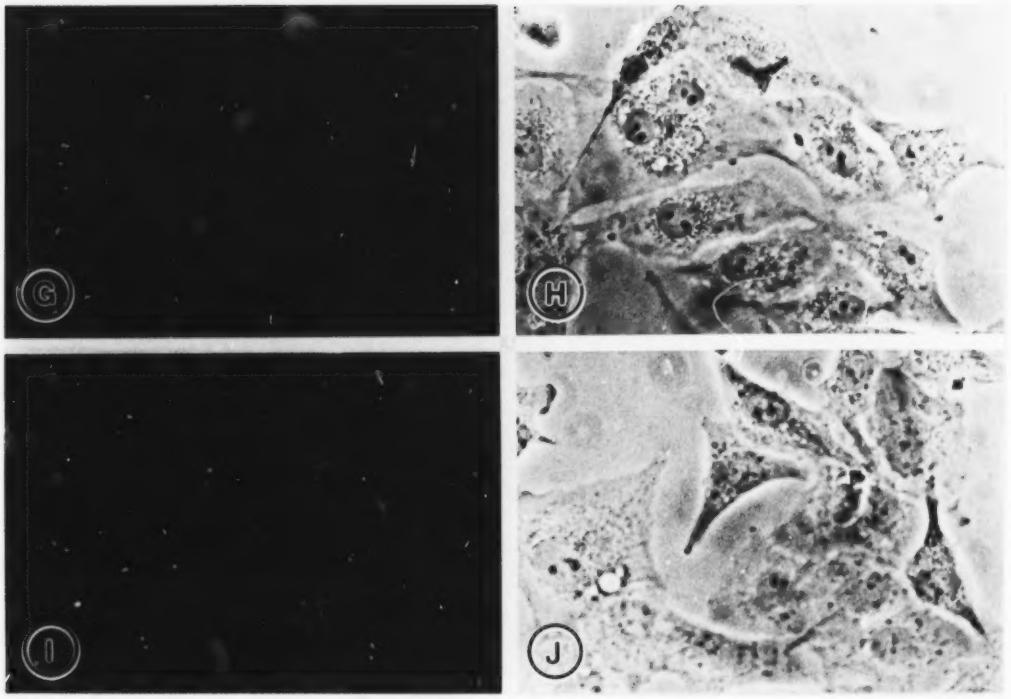
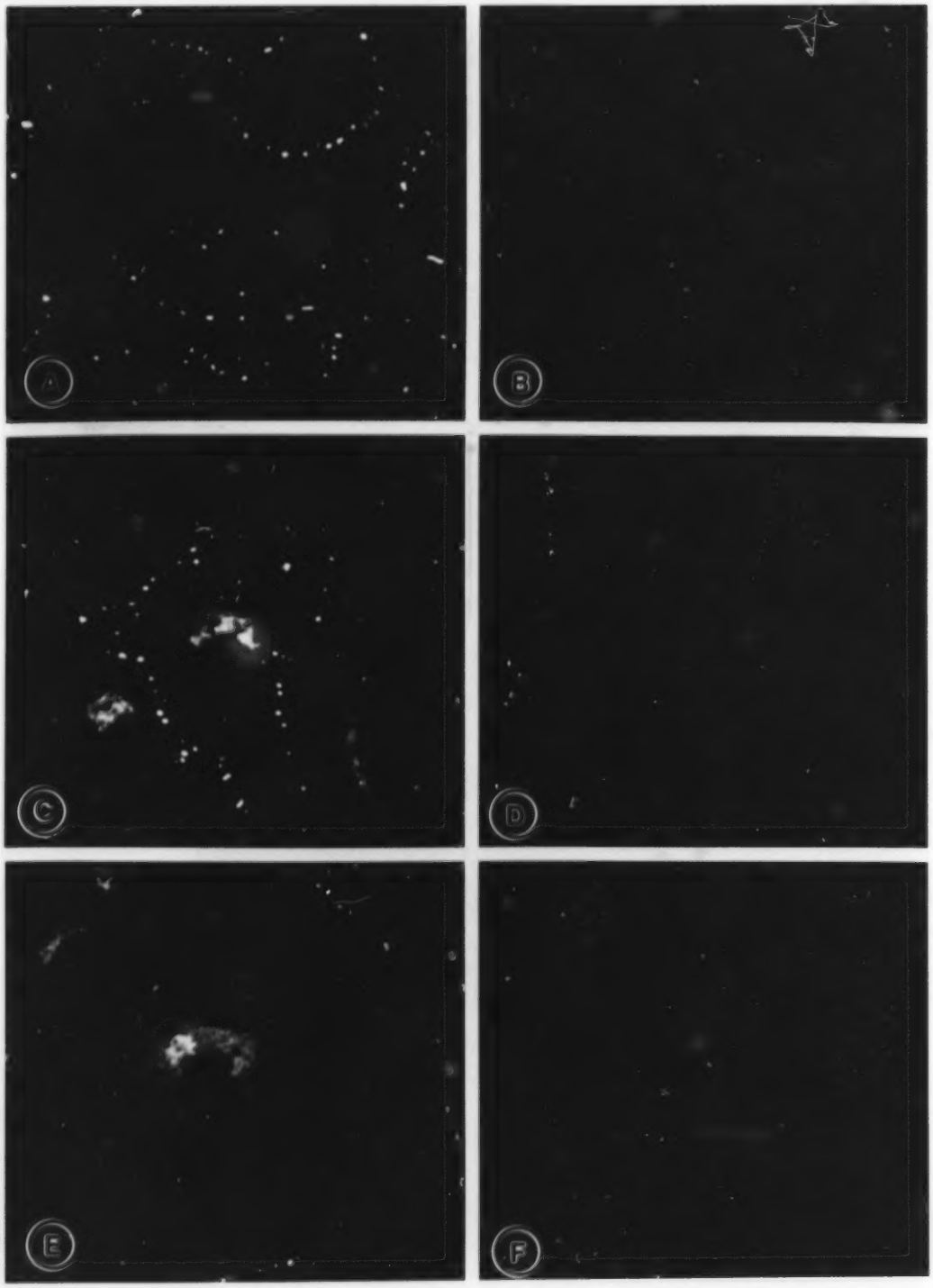

G
H
I
J

Fig. 6.4. Confocal laser scanning microscopy images of immunofluorescence labeled AB5 cells after zinc treatment. Cells were treated with zinc acetate, and immuno-labeled with α_1s antibodies after 6, 12, 24, and 36 hours of the treatment. Figures A, C, E, G, and I show the immunofluorescence labeling of, respectively, untreated, 6-, 12-, 24-, and 36 hours of treatment. Figures B, D, F, H, and J are corresponding controls which were labelled with pre-immune serum. Magnification= 600x.


A
B
C
D
E
F

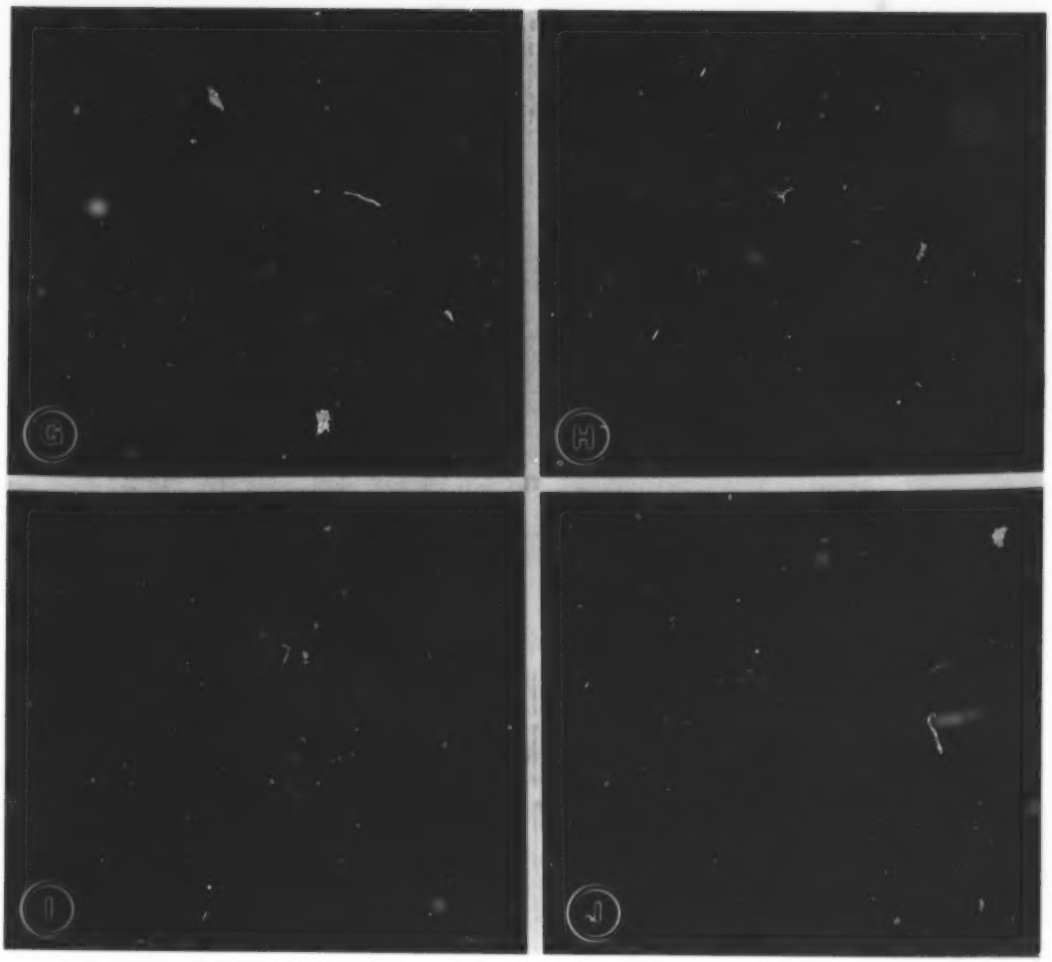

G
H
I
J

Fig. 6.5. Confocal laser fluorescence scanning images of immunolabelling of AB5 cells for connexin43 after 24 hours of zinc and cadmium treatment. Cells were treated with zinc acetate and cadmium chloride, and immunolabelled with α_1s antibodies. (A) AB5 cells labeled with α_1s without treatment, (B) AB5 cells labeled with pre-immune serum without treatment, (C) AB5 cells labelled with α_1s after zinc and cadmium treatment, and (D) AB5 labelled with pre-immune serum after zinc and cadmium treatment.

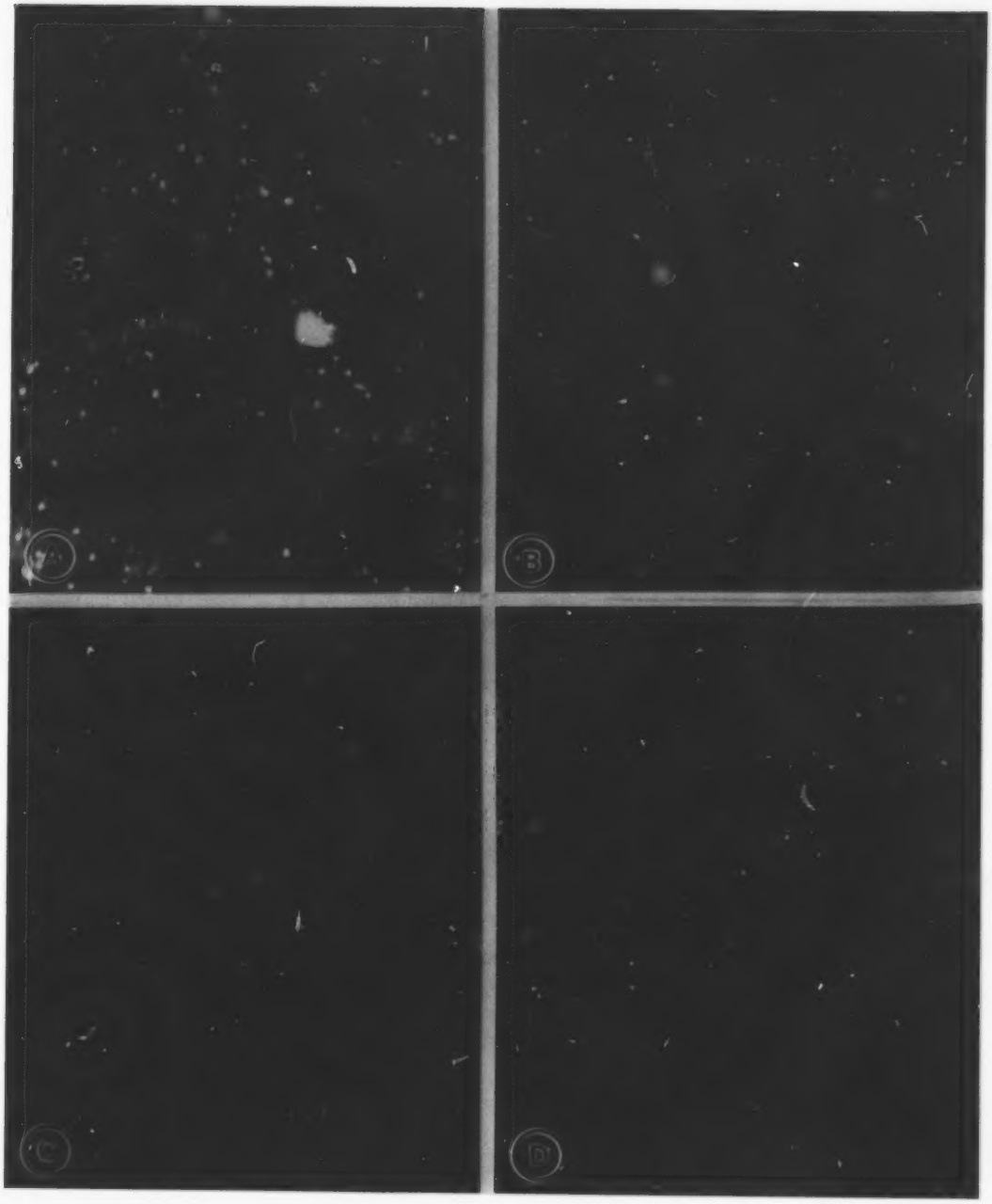
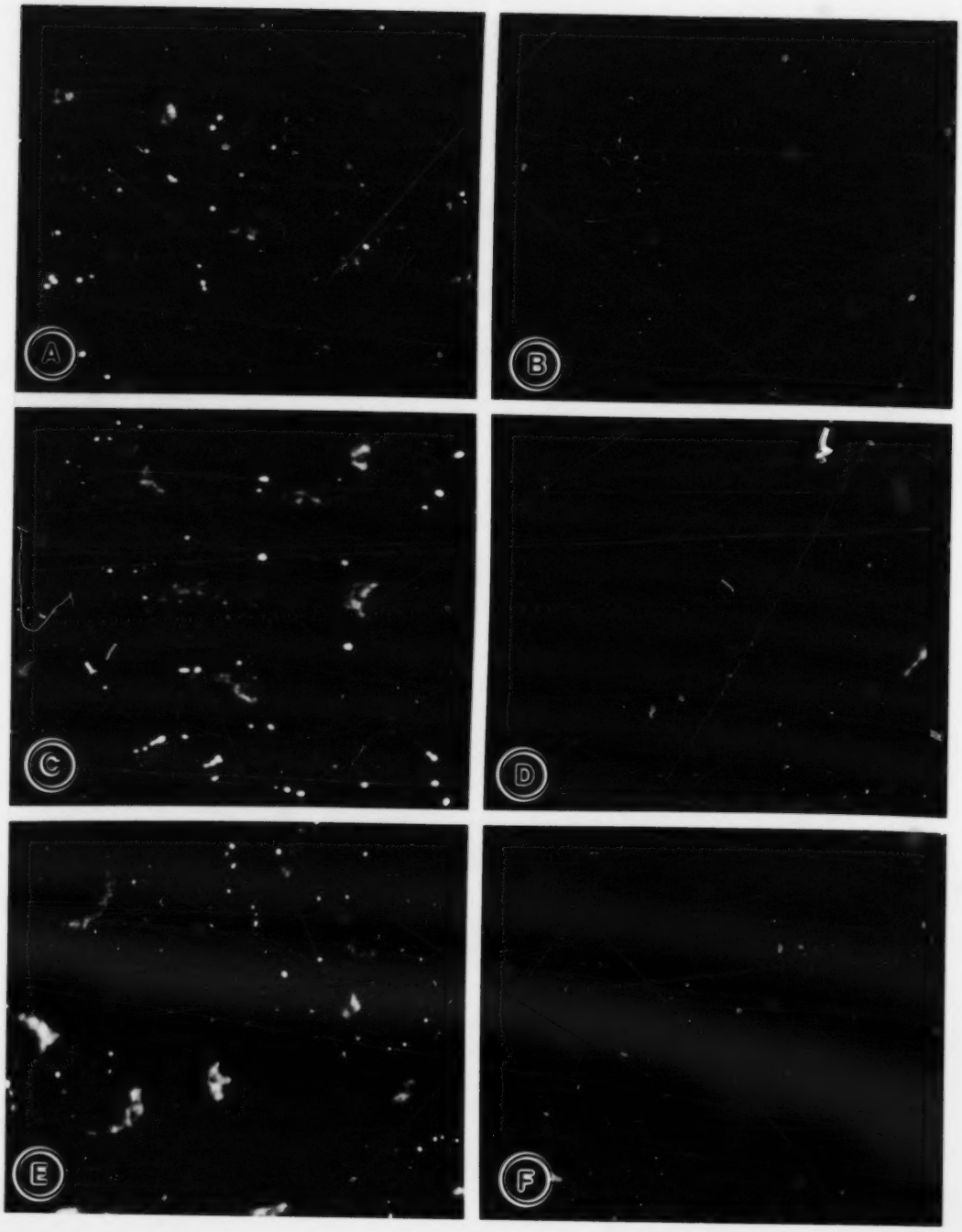
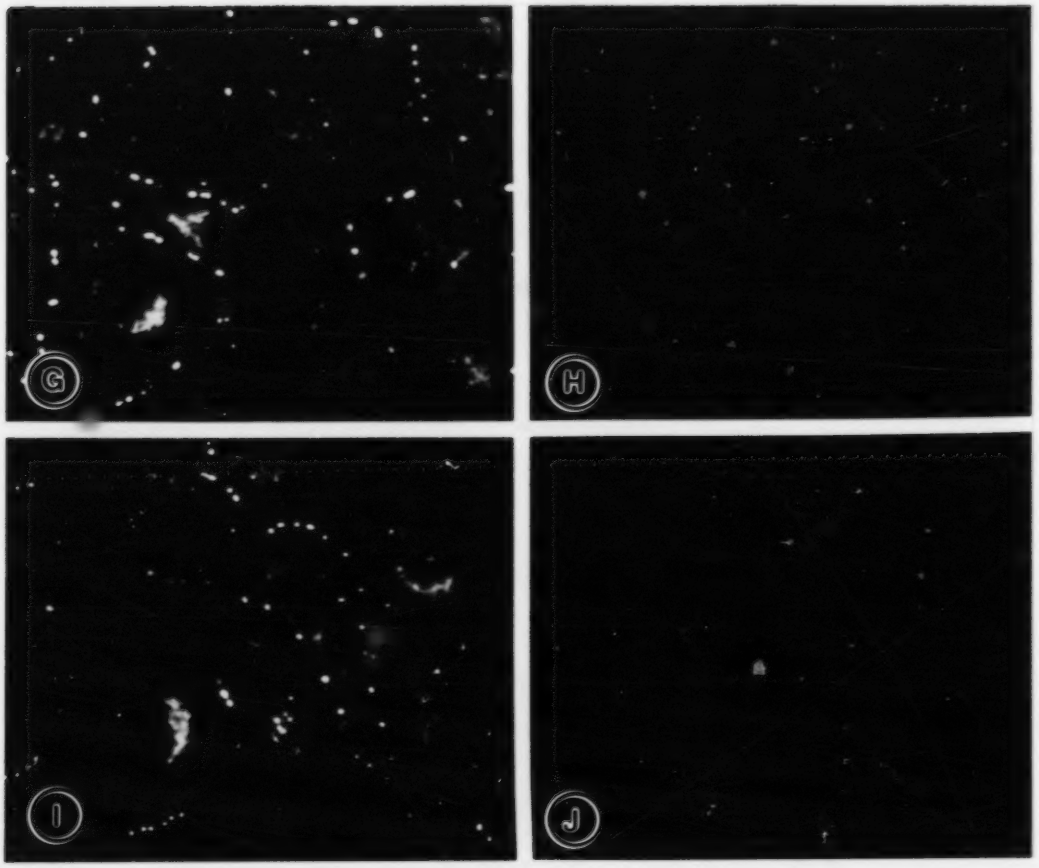

A
B
C
D

Fig. 6.6. Confocal laser fluorescence scanning images of immunolabeling of 9L cells for connexin43 after zinc treatment. Cells were treated with zinc acetate, and immunolabeled with α_1s antibodies. Figures A, C, E, G, and I show the immunofluorescence labeling of, respectively, untreated, 6-, 12-, 24- and 36 hours of treatment. Figures B, D, F, H, and J are corresponding controls which were labeled with pre-immune serum. Magnification= 600x.


A
B
C
D
E
F


G
H
I
J

Quantitation of the punctate spots was done on confocal images of immunolabeled cells. Images from at least four randomly selected areas on the coverslips were obtained by confocal microscopy at each of the above mentioned time points. Gap junctional punctate spots were counted in a given number of cells in the image area. The results are presented as the number of gap junctional plaques per cell, and the average size of the plaques is also given (Table. 6.1). The results show that treatment of 9L cells with zinc acetate did not result in appreciable change either in the mean number of gap junctions per cell or in the average size of the gap junctional plaques in these cells. In AB5 cells, on the other hand, the effects of zinc acetate treatment were drastic. Within a short time after the treatment (6 hours), both the mean number of gap junctions per cell (Fig. 6.7) and the average size of the gap junctions (Fig. 6.8) started to decrease. After 24 hours of zinc treatment, the mean number of gap junctions per cell and the average size of the gap junctions were the lowest. The reduction in these parameters was seen even after 36 hours of treatment. These results clearly show that treatment of AB5 cells with zinc acetate resulted in a large decrease in the number and the size of gap junctions whereas untransfected 9L cells did not show an appreciable diminution in either of these parameter (Fig. 6.7 and 6.8).

Immunoblotting of proteins from zinc treated cells

Decrease in gap junctions in AB5 cells would be expected to be due to decrease in connexin43. Antisense RNA would be expected to inhibit Cx43 expression by affecting at both pre- and post-transcriptional events. The decrease in connexin43 was determined on immunoblots of the cell homogenates. When equal amounts of proteins from whole cell homogenates of zinc treated and untreated AB5 cells were blotted on nitrocellulose and probed with α_1s antibodies, the level of connexin43 protein was found to decrease after zinc treatment (Fig. 6.9). A gradual decrease in the level of connexin43 band was seen after 12 hours of addition of zinc into the culture media (Fig. 6.9 lane D) and continued until 36 hours after treatment (lane F), when the connexin band was not detectable. Immunoblotting of total cell protein from zinc treated untransfected 9L cells did not show a decrease in connexin43 protein, even after 18 hours of treatment (Fig. 6.10).

Taken together, these results from immunofluorescence labeling and immunoblotting experiments demonstrate that treatment of transfected AB5 cells with

Table.6.1. Effect of zinc treatment on the number and size of gap junctions between cells of 9L and AB5 cultures. After immunofluorescence labeling with α1s antibodies, number of gap junction fluorescence spots between cells and their size were determined from confocal laser images of 9L and AB5 cells. The table represents data from at least three such images for each treatment. The numbers in the parentheses represent the standard deviation of the data.

Treatment	Average number of gap junctions per cell		Average size of gap junctions (μm)	
	9L Cells	AB5 cells	9L cells	AB5 cells
No zinc	10.88 (+/- 0.78)	12.46 (+/- 0.63)	1.77 (+/- 0.05)	1.32 (+/- 0.15)
6 hours zinc treatment	10.77 (+/- 2.29)	6.09 (+/- 1.04)	1.69 (+/- 0.35)	0.95 (+/- 0.37)
12 hours zinc treatment	12.25 (+/- 1.05)	4.17 (+/- 0.69)	1.37 (+/- 0.11)	0.84 (+/- 0.24)
24 hours zinc treatment	11.63 (+/- 1.69)	1.92 (+/- 0.36)	1.46 (+/- 0.12)	0.52 (+/- 0.12)
36 hours zinc treatment	11.50 (+/- 0.78)	3.07 (+/- 1.09)	1.31 (+/- 0.14)	0.73 (+/- 0.10)

Fig. 6.7. Effect of antisense induction by zinc treatment on the number of gap junctions in 9L and AB5 cells. Number of the immunofluorescent connexin43 spots was counted from at least four confocal image frames of untransfected 9L and transfected AB5 cells. Vertical bars display one standard deviation.

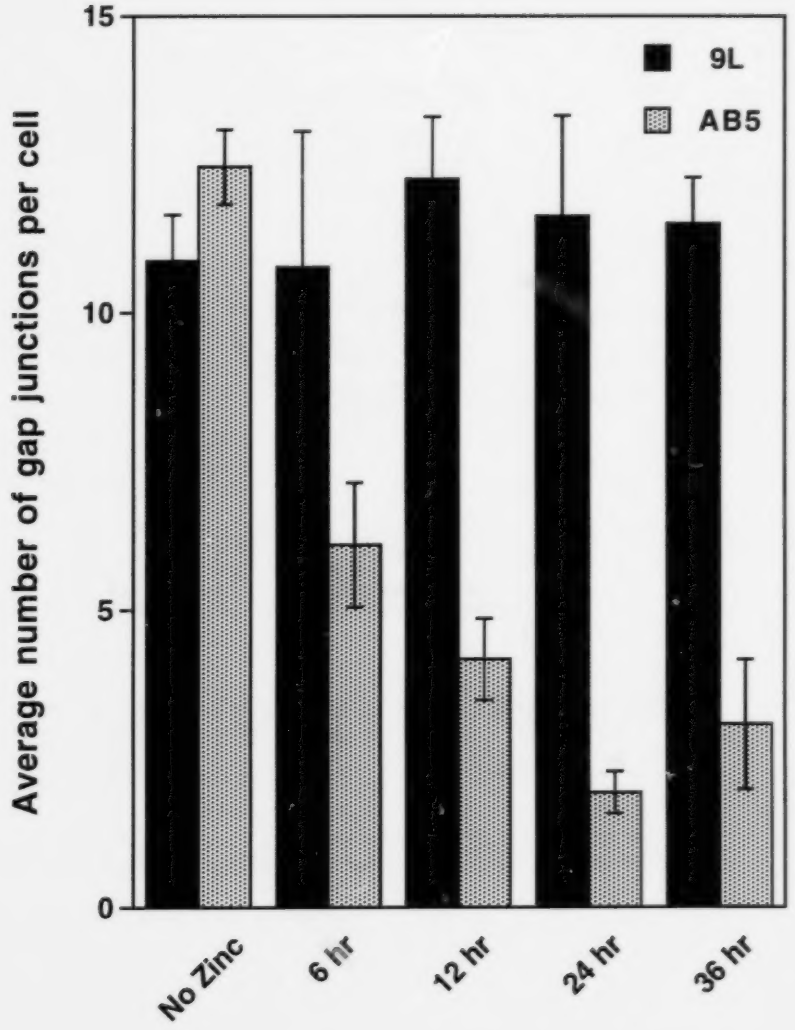

Average number of gap junctions per cell
15
10
5
0
9L
AB5
No Zinc
6 hr
12 hr
24 hr
36 hr

Fig. 6.8. Effect of antisense induction by zinc treatment on the size of immunolabeled gap junction spots of 9L and AB5 cells. Size of the immunofluorescent connexin43 spots was measured from at least four confocal image frames of untransfected 9L and transfected AB5 cells. Vertical bars display one standard deviation.

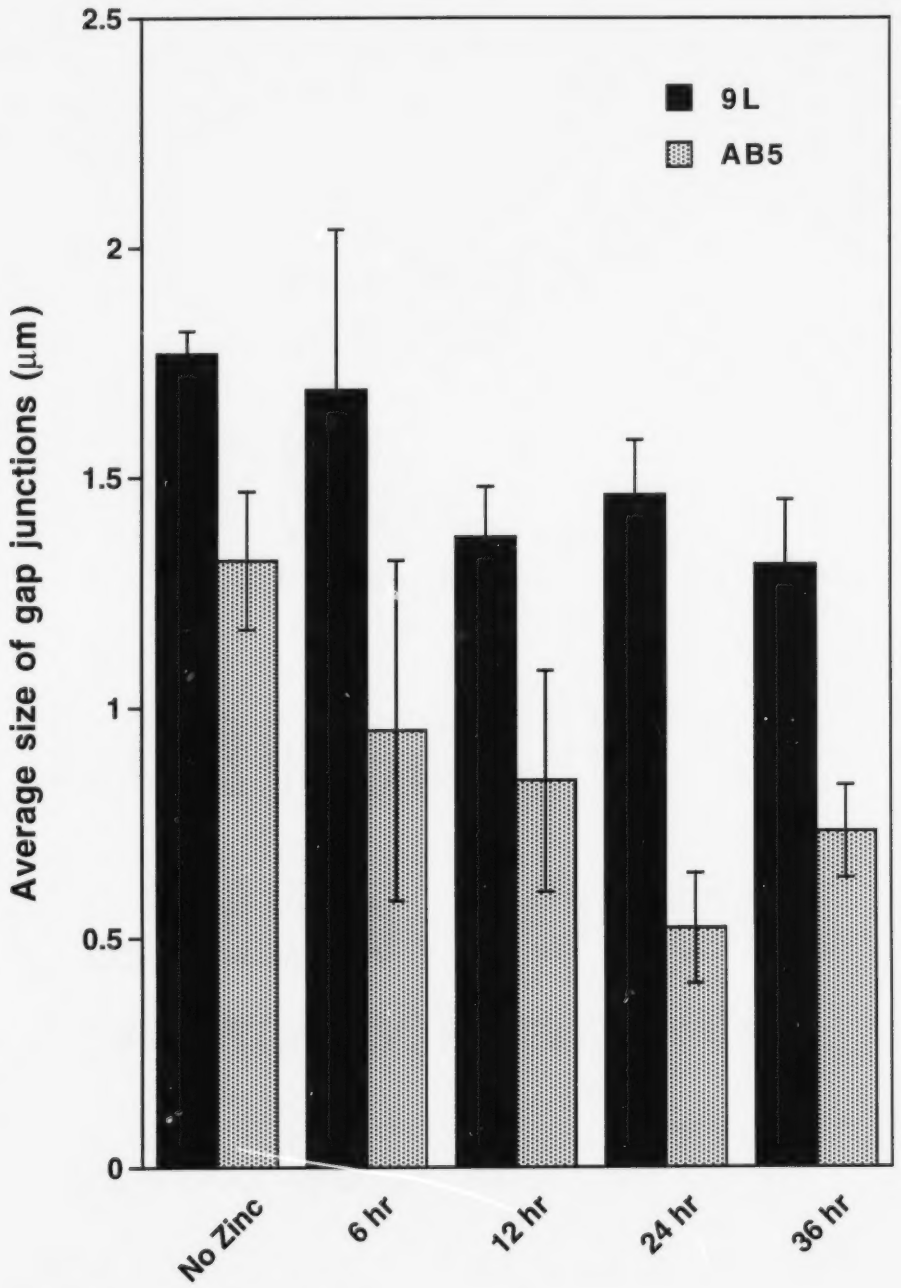

2.5
2
1.5
1
0.5
0
Average size of gap junctions (μm)
9L
AB5
No Zinc
6 hr
12 hr
24 hr
36 hr

Fig. 6.9. Immunoblotting of crude cell homogenates of AB5 cells after zinc treatment. The upper figure shows the connexin43 protein bands in cell homogenates after probing with α_1s antibodies; the lower figure shows the Ponceau-S stained protein blot before probing. Lanes are marked as follows; (A) molecular weight markers, (B) untreated AB5, (C) 6 hours treatment, (D) 12 hours treatment, (E) 24 hours treatment, and (F) 36 hours treatment. The numbers at the left indicate the size of molecular weight markers in kilodaltons.

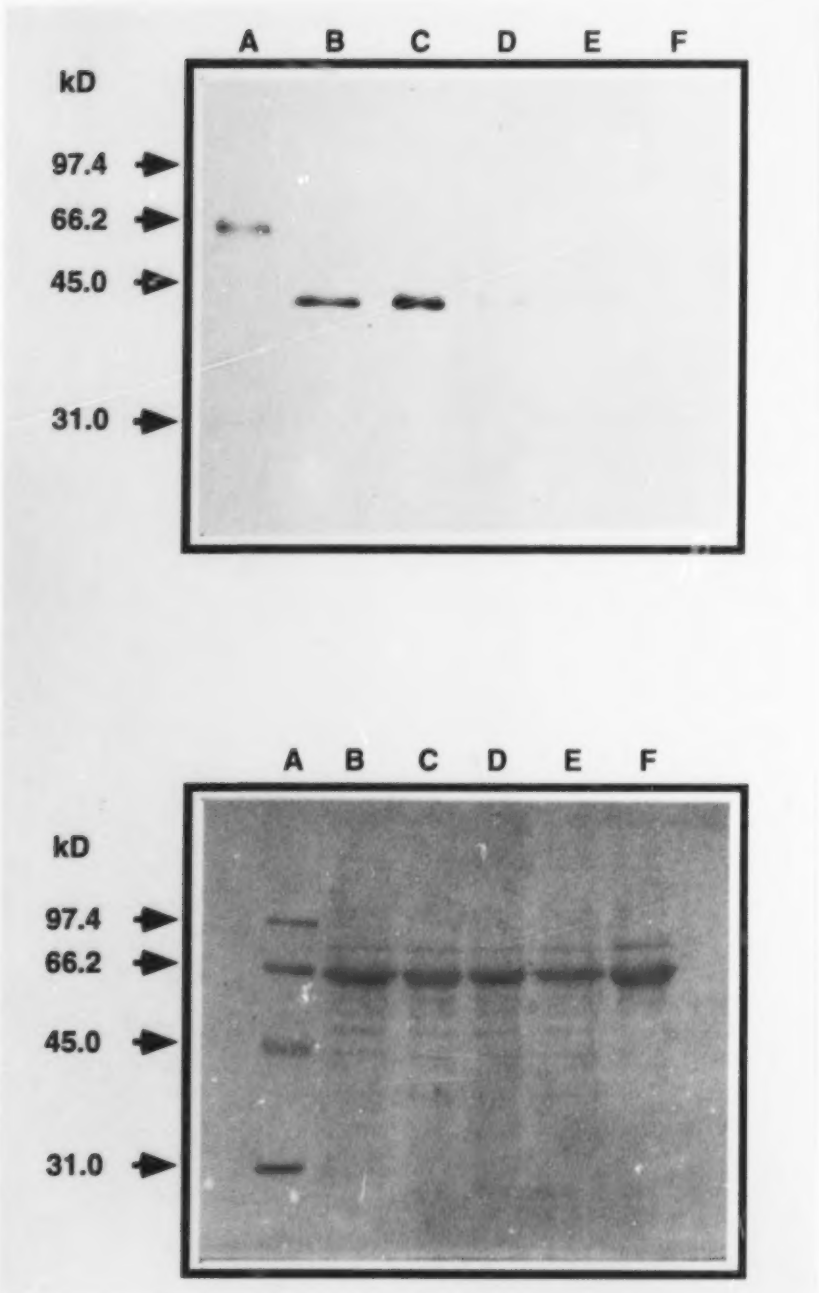
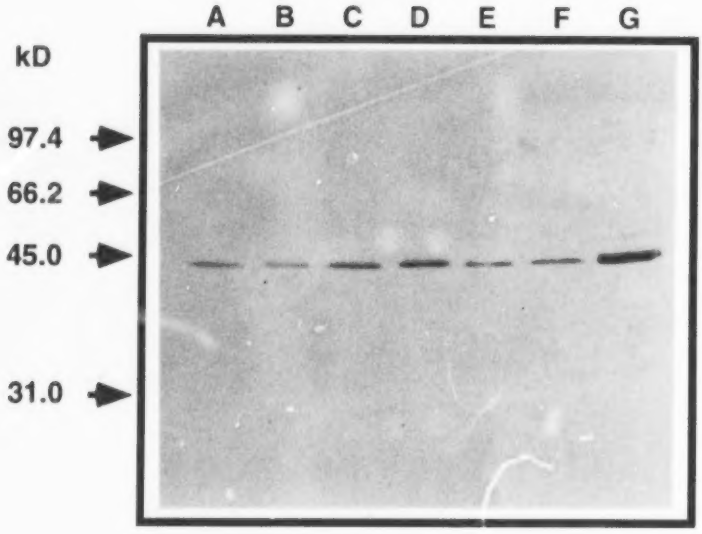

A B C D E F
kD
97.4
66.2
45.0
31.0
A B C D E F
kD
97.4
66.2
45.0
31.0

Fig. 6.10. Immunoblotting of crude cell homogenates of 9L cells after zinc treatment. Lanes are marked as follows; (A) untreated 9L, (B) 3 hours treatment, (C) 6 hours treatment, (D) 9 hours treatment, (E) 12 hours treatment, (F) 18 hours treatment, and (G) rat heart protein sample as a positive control. The numbers at the left indicate the size of molecular weight markers in kilodaltons.


A B C D E F G
kD
97.4
66.2
45.0
31.0

zinc acetate resulted in decrease of both the amount of connexin43 protein and the number of gap junctions between adjacent cells.

Expression of sense and antisense connexin RNA

Northern blot analyses

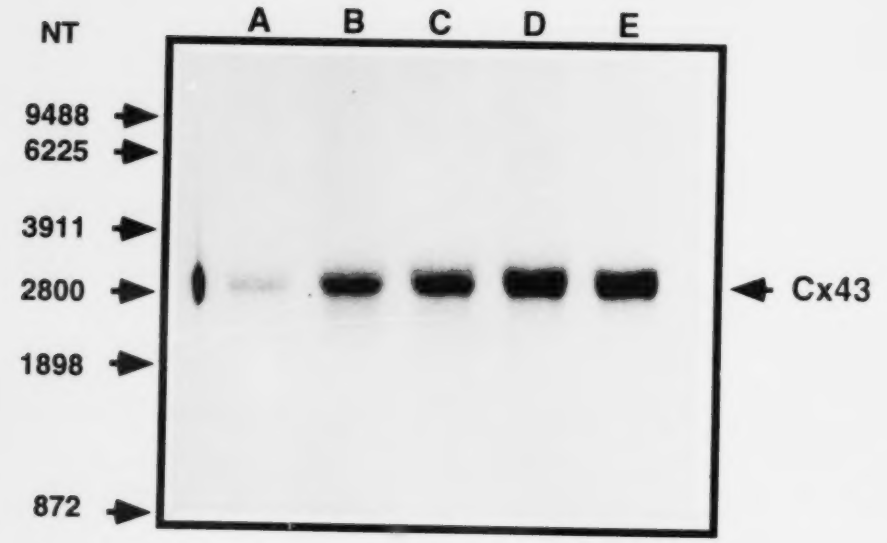
Antisense connexin43 RNA may produce its effects in several ways (Kim *et al.*, 1985; Kimelman, 1992). Expression of antisense RNA might either cause rapid degradation in sense RNA for connexin43, or it might inhibit translation, or both. If the sense RNA susceptibility to endogenous RNases is increased, then a decrease in the levels of mRNA should be expected. Expression of sense connexin43 RNA in the untransfected 9L cells and transfected AB5 cells was examined by Northern blot analyses. Antisense RNA expression was determined by RNase protection assays because of the higher sensitivity of this technique.

After adding zinc acetate to the cell cultures, total cellular RNA was isolated from cells at 6, 12, 24 and 36 hours as well as from untreated cells. Probing the RNA blots of untransfected 9L cells with radiolabeled Cx43 probe showed a single RNA band in all of the lanes (Figure 6.11). The level of Cx43 mRNA expression in 9L cells, after zinc acetate treatment, increased gradually. The loading of RNA samples on the gel was comparable as judged by the intensity of β-actin RNA in the different lanes. AB5 RNA blots, on the other hand, did not show a gradual increase after zinc acetate treatment (Fig. 6.12). The RNA band intensities at different time points after zinc treatment showed an increase followed by a relatively stable expression. Even with the use of nick-translated probes, that contain both sense and antisense strands of probes, an antisense RNA band was not detected. Abundance of β-actin mRNA on the Northern blots was taken as loading control in these experiments.

RNase protection assays for detection of antisense Cx43 RNA

RNase protection assays were performed to detect the expression of antisense RNA in AB5 cells (Fig. 6.13) because RNase protection assays are more sensitive than Northern analyses and the method can detect smaller RNA fragments. RNA samples from AB5 cells either without zinc acetate treatment, or after 6, 12, 24, and 36 hours of zinc treatment were hybridized with radioisotope-labeled sense RNA probe. A yeast RNA sample (Fig. 6.13, lane B), and an untransfected 9L RNA sample (lane C) were used

Fig. 6.11. Northern blot analyses of 9L RNA with Cx43 and β-actin probes. The upper figure shows the 9L blot probed with Cx43 probe, and the lower figure shows the same blot probed with mouse β-actin probe. Samples in the lanes are; (A) RNA from untreated 9L cells, (B) RNA from 6 hour treatment, (C) RNA from 12 hour treatment, (D) RNA from 24 hour treatment, and (E) RNA from 36 hours treatment. Arrows at the left indicate the size of RNA markers in nucleotides (NT).


NT
A
B
C
D
E
9488
6225
3911
2800
1898
872
Cx43

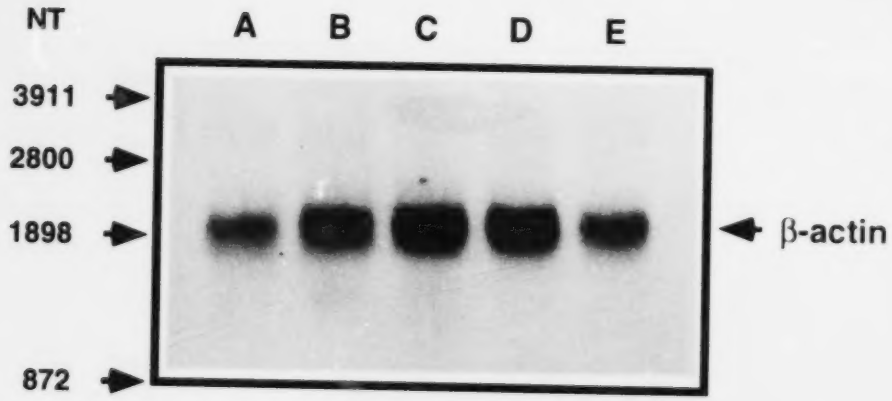
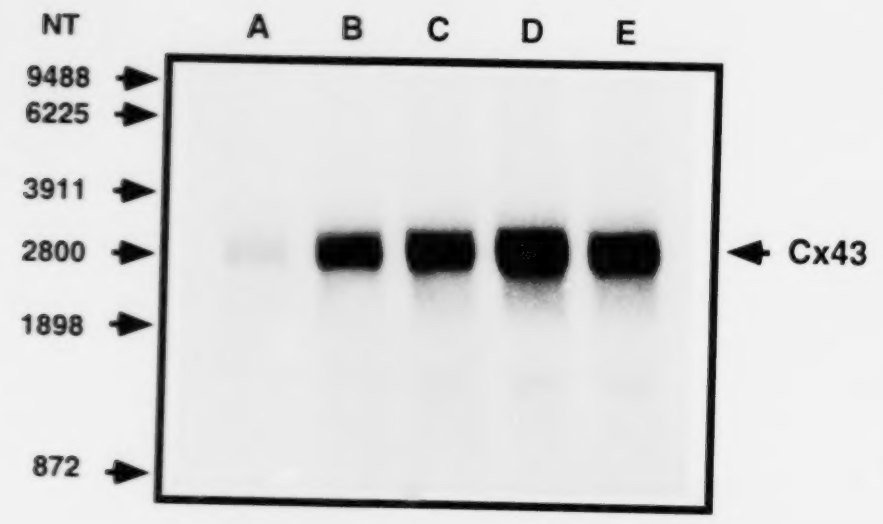

NT
A
B
C
D
E
3911
2800
1898
872
β-actin

Fig. 6.12. Northern blot analyses of AB5 RNA with Cx43 and β-actin probes. The upper figure shows the AB5 blot probed with Cx43 probe, and the lower figure shows the same blot probed with mouse β-actin probe. Samples in the lanes are; (A) RNA from untreated AB5 cells, (B) RNA from 6 hour treatment, (C) RNA from 12 hour treatment, (D) RNA from 24 hour treatment, and (E) RNA from 36 hours treatment. Arrows at the left indicate the size of RNA markers in nucleotides (NT).


NT
A
B
C
D
E
9488
6225
3911
2800
1898
872
Cx43

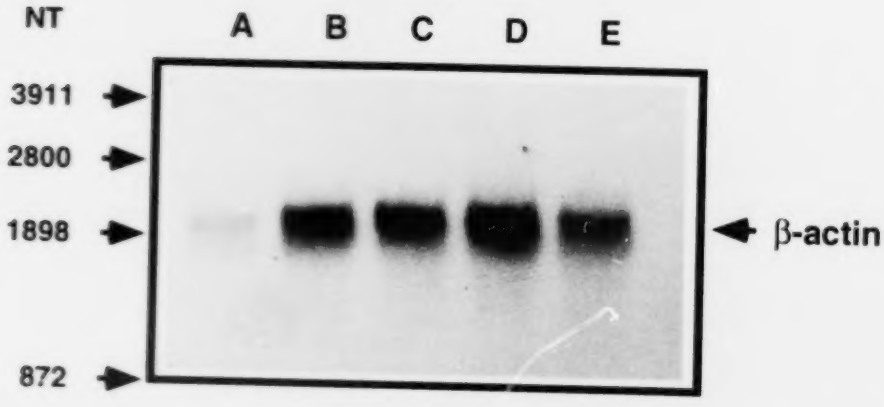
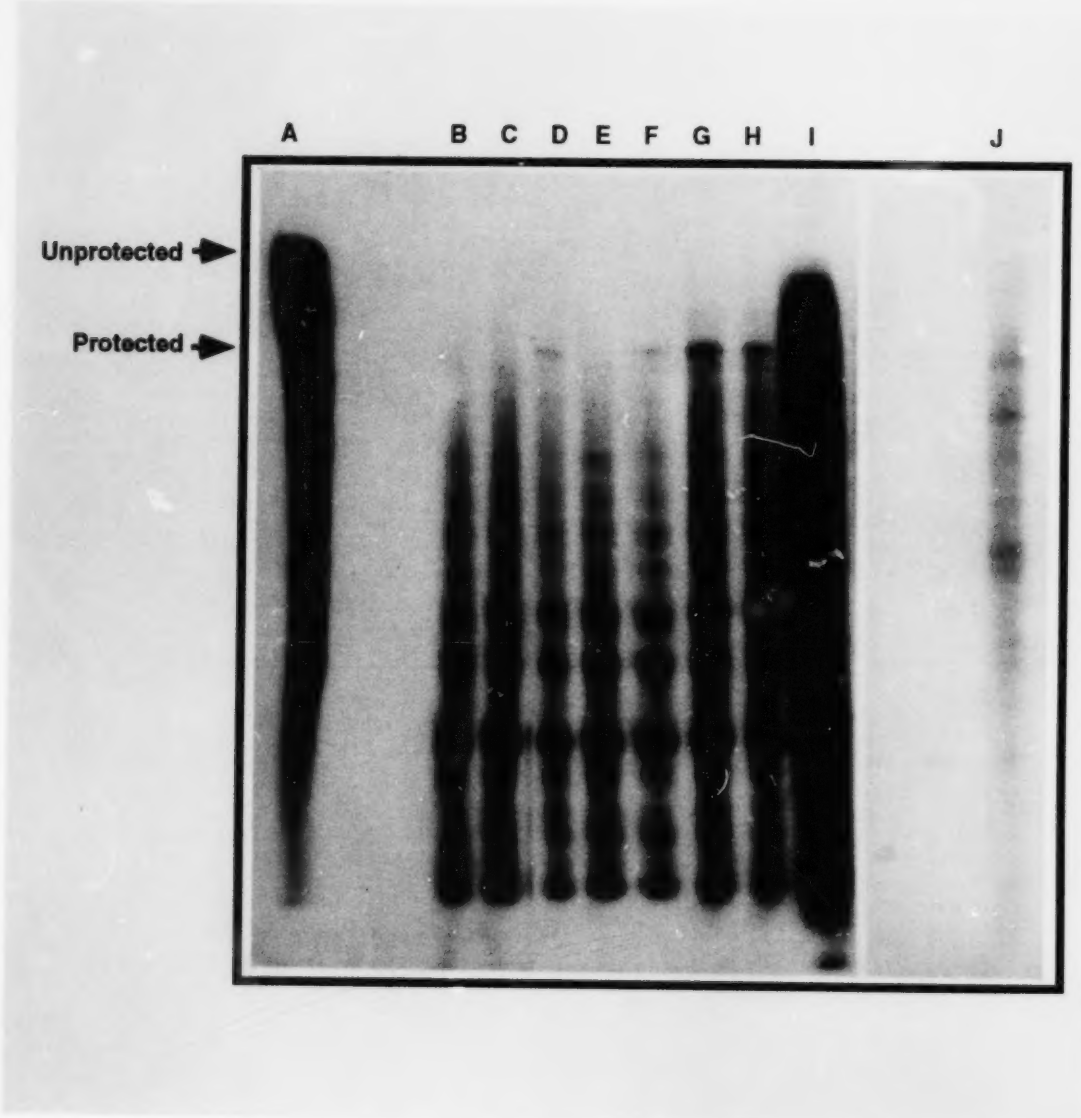

NT
A
B
C
D
E
3911
2800
1898
872
β-actin

Fig. 6.13. RNase protection assay for antisense RNA detection in zinc treated AB5 cells. Lane A shows the full-length probe without RNase treatment. Lanes B to H are RNase treated which contain RNA samples from, respectively; (B) Yeast RNA, (C) untransfected 9L, (D) untreated AB5, (E) 6 hours treatment, (F) 12 hours treatment, (G) 24 hours treatment, and (H) 36 hours treatment. Lanes I and J are of the same sample (*in vitro* synthesized antisense Cx43 RNA) at different exposure; in order to show the bands clearly, lane J was exposed for shorter duration. The antisense RNA band was protected by hybridization to the probe and, therefore, is shorter in length (arrowhead).


A
B C D E F G H I
J
Unprotected
Protected

as negative controls. A sample of *in vitro* synthesized antisense RNA (lane J) was used as a positive control for RNase protection. In four such experiments, the expression of antisense RNA was detected in AB5 RNA samples after zinc acetate treatments. The *in vitro* synthesized sense strand positive control showed the protected fragment as did the RNA samples from zinc treated AB5 cells (Fig. 6.13; lanes D, E, F, G, and H). The lack of bands in the lanes containing yeast RNA (lane B) and untransfected 9L RNA (lane C) validated the presence of protected fragments. *In vitro* synthesized antisense connexin43 RNA sample showed the same protected band as in the transfected AB5 RNA samples (lane J), confirming the validity of the protected bands in these samples.

Gap junctional communication in the transfected cells after antisense RNA expression

In order to assess the effect of antisense connexin43 RNA expression on the intercellular communication between transfected cells, the scrape loading/dye transfer method was used. The results obtained after 24 hours of zinc acetate treatment of AB5 cells are shown in Fig. 6.14. As is evident from this Figure, after zinc treatment Lucifer Yellow CH dye did not diffuse to neighboring cells (Fig. 6.14 C), whereas the dye diffused to neighboring cells in untreated cultures (Fig. 6.14 A). To determine if this reduction in intercellular communication was due to the effect of zinc treatment, untrasfected 9L cells were also monitored for dye transfer with or without zinc acetate treatment. The results in Fig. 6.15 A and C show that dye transfer in 9L cells was unaffected by zinc treatment. Thus, antisense RNA expression in the AB5 cells reduced intercellular gap junctional communication.

Together, these results showed that the transfection of 9L cells with antisense RNA synthesizing plasmid was successful, and that multiple copies of the antisense plasmid construct were present in the transfected AB5 cells. Antisense RNA expression by zinc treatment was observed by RNase protection assays. Induction of antisense RNA by zinc treatment resulted in a decrease in both connexin43 protein and in the number and size of gap junctions between these cells, which in turn resulted in reduced dye transfer.

Fig. 6.14. Scrape loading/ dye transfer of Lucifer Yellow CH fluorescent dye for intercellular dye transfer in AB5 cells. (A) intercellular dye transfer between untreated AB5 cells, (B) phase-contrast micrograph of the same region, and (C) dye transfer in AB5 cells after 24 hours of zinc treatment, (D) corresponding phase-contrast micrograph. Magnification= 400x.

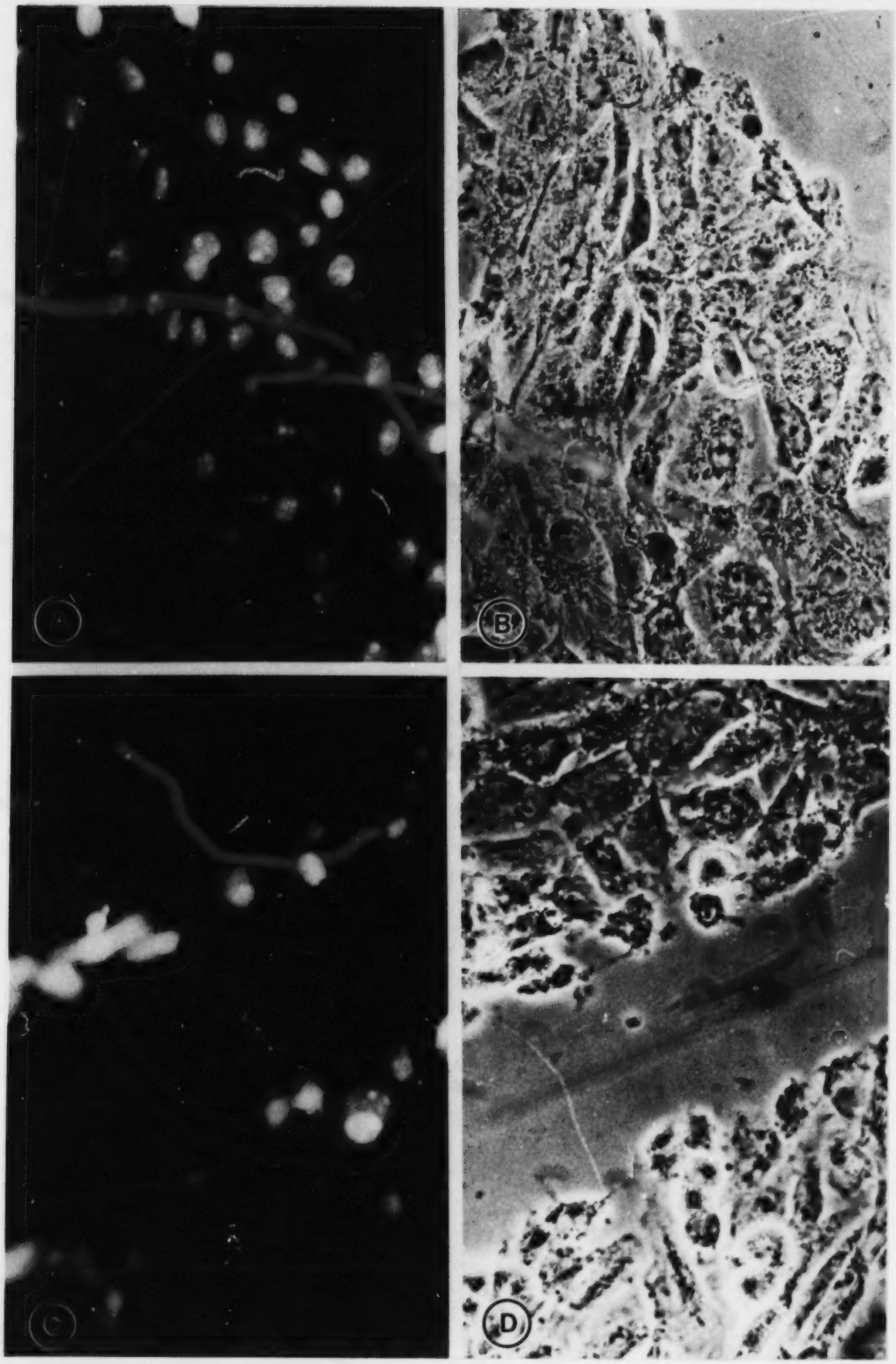
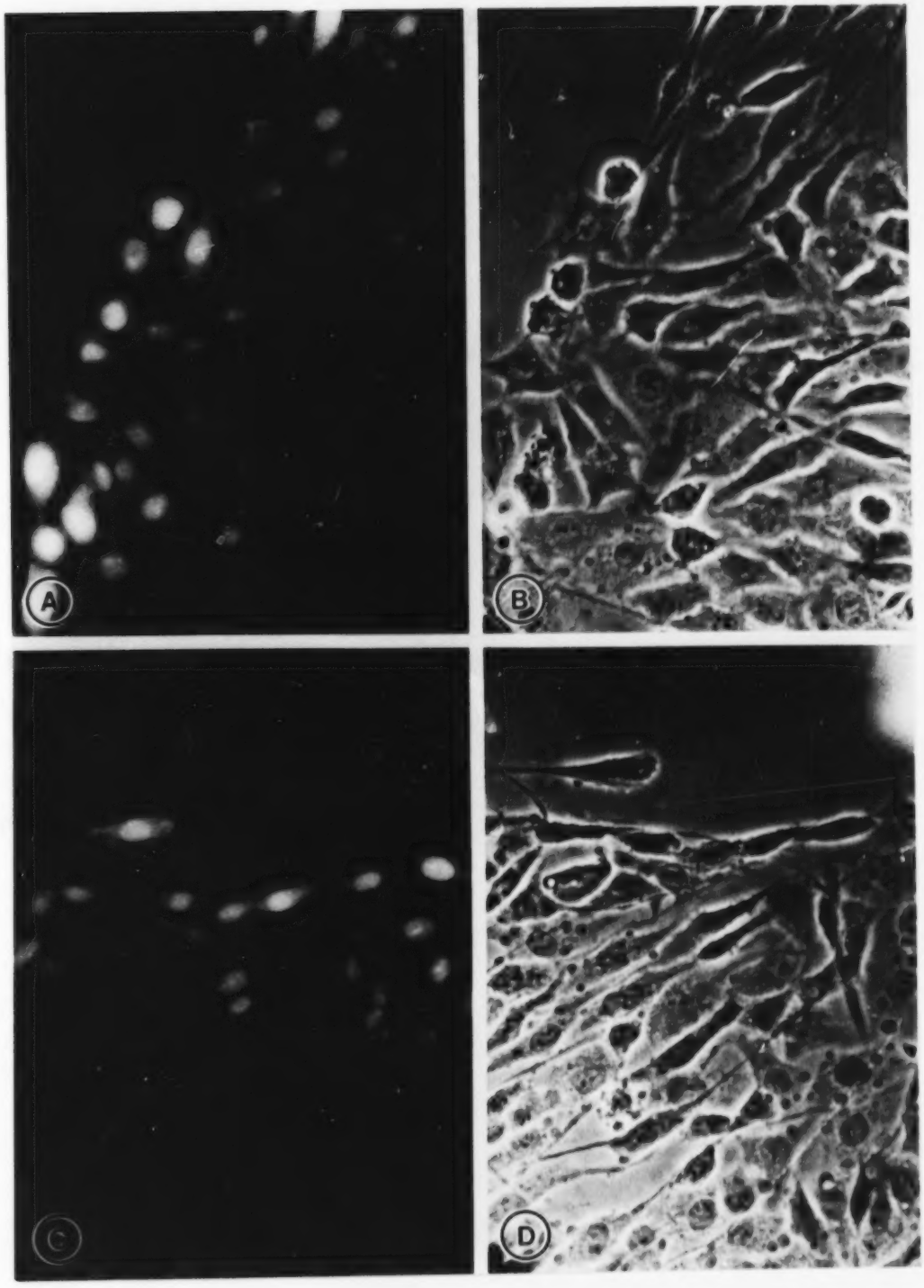

A
B
C
D

Fig. 6.15. Scrape loading/ dye transfer of Lucifer Yellow CH fluorescent dye for intercellular dye transfer in untransfected 9L cells. (A) intercellular dye transfer between untreated 9L cells, (B) phase-contrast micrograph of the same region, and (C) dye transfer in 9L cells after 24 hours of zinc treatment, (D) corresponding phase-contrast micrograph. Magnification= 400x.


A
B
C
D

Comparison of growth rates of transfected and untransfected cells

To assess the effect of inhibition of gap junctions on the growth of transfected cells, the untransfected 9L cells and transfected AB5 cells were grown in absence or presence of zinc acetate (75 μM) in the growth medium. Cells from three petri-dishes of each group were counted at daily intervals in triplicates over six days and growth curves were plotted. Growth curves for untransfected 9L cells were also plotted from cells grown in absence and in the presence of zinc acetate. The results obtained are shown in the Figure 6.16. The population doubling time of cells in these cultures was calculated during the log growth phase. The population doubling time for AB5 cells in cultures without zinc acetate was 22.36 hours, whereas in the cultures grown in presence of 75 μM zinc acetate the population doubling time was 27.73 hours. For the untransfected 9L cells, the population doubling time for cells in absence and in the presence of zinc acetate were calculated to be 15.40 hours and 16.12 hours, respectively.

Multiple regression analyses of the growth of cells during the log growth phase (day ≥ 3) indicated that the goodness of fit for different lines was better than 0.97 (Fig. 6.17). Also, the regression analyses indicated that the slopes of the lines for untreated and zinc acetate-treated 9L cells were not significantly different ($p=0.8953$). However, the slopes of the untreated and zinc acetate-treated AB5 transfectants were found to be significantly different ($p=0.0046$). A comparison of cell counts at day 6 by *t-test* also showed a significant difference between untreated and zinc-acetate treated AB5 cells. In addition, the slopes of the lines for untreated 9L and untreated AB5 cells were significantly different ($p=0.0031$).

Thus, the growth of transfected AB5 cells was affected by the presence of zinc, that showed significant retardation of the growth. This retardation is possibly due to the induction of antisense Cx43 RNA synthesis. Zinc acetate treatment did not significantly affect the growth rate of untransfected 9L cells. A considerable difference was observed between the population doubling times of untransfected 9L and transfected AB5 cells without treatment. This difference could possibly be due to transfection itself.

Fig. 6.16. Comparison of growth of untreated and zinc-treated 9L and AB5 cells in culture. The cells were grown in triplicates and were counted daily. Graph was plotted on a semi-logarithmic scale with the Y-axis being logarithmic. Vertical bars represent one standard deviation.

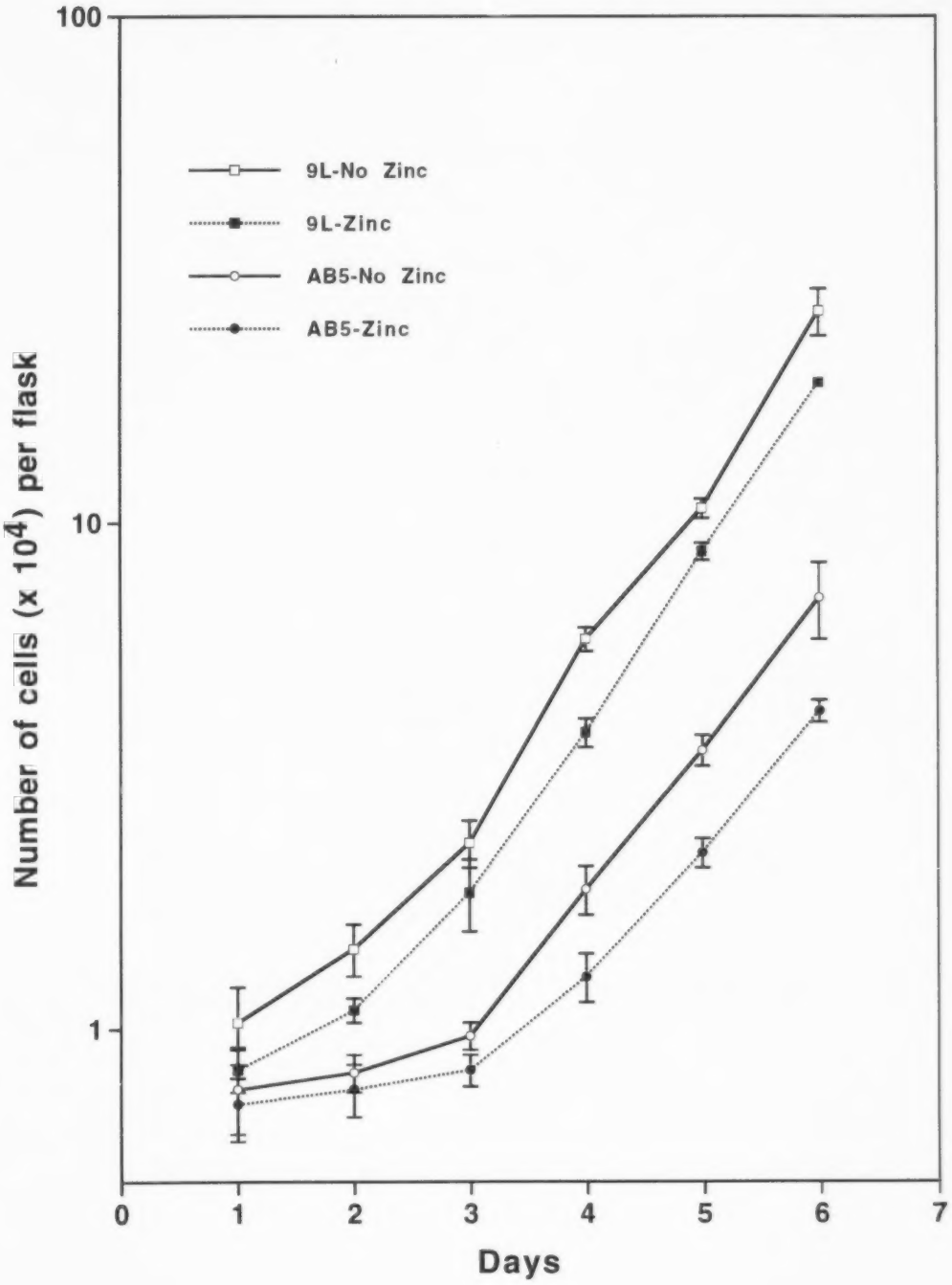
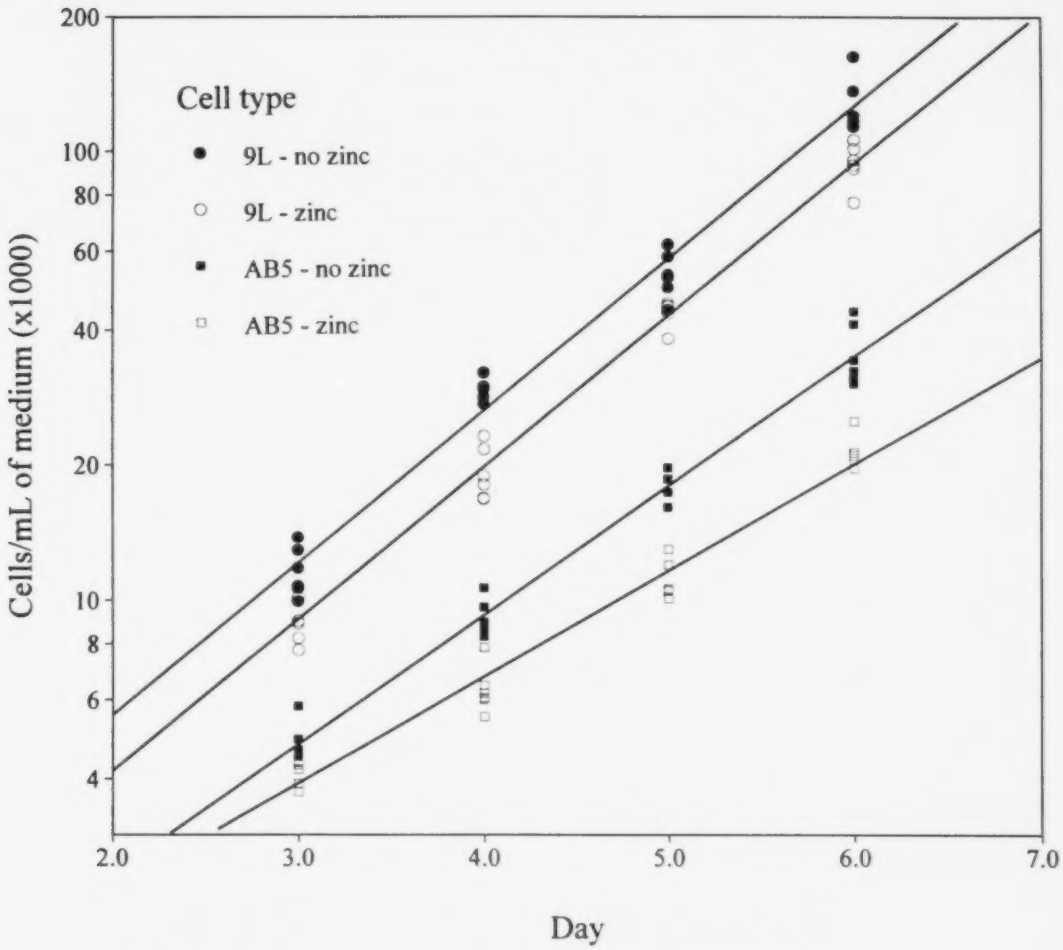

100
10
1
Number of cells (x 10^4) per flask
9L-No Zinc
9L-Zinc
AB5-No Zinc
AB5-Zinc
0
1
2
3
4
5
6
7
Days

Fig. 6.17. Multiple regression analysis for the growth rates of untreated and zinc acetate treated 9L and AB5 cells. The goodness of fit (r^2=)for the lines for untreated 9L, zinc treated 9L, untreated AB5, and zinc treated AB5 cells (transfected) were 0.9781, 0.9839, 0.9818, and 0.9712, respectively. The slopes of the lines (growth rate) for untreated and zinc treated AB5 cells were significantly different (p= 0.0046) whereas those for untreated and zinc treated 9L cells (untransfected) were not different (p= 0.8953). Also, the slopes of untreated 9L and untreated AB5 were different (p= 0.0031).


Cell type
9L - no zinc
9L - zinc
AB5 - no zinc
AB5 - zinc
Cells/mL of medium (x1000)
200
100
80
60
40
20
10
8
6
4
2.0
3.0
4.0
5.0
6.0
7.0
Day

Chapter 7

DISCUSSION

The present study was carried out with the following objectives: (a) to determine the suitability of 9L rat glioma cells as an in vitro model for research on gap junctions in glioma cells, and (b) to determine whether gap junctions play a major role in the regulation of growth in glioma cells. The results obtained from this study establish that 9L cells can be used as a model cell line for studying gap junctions in glioma cells. The results show that gap junctions may play a role in the regulation of growth (proliferation) of glioma cells *in vitro*, although this role may not be the major one.

Comparison of gap junctions in 9L and C6 rat glioma cells

The 9L rat glioma cell line was analyzed for gap junction formation, and compared with the C6 rat glioma cell line. The results show that 9L cells form more communicating gap junctions than C6 cells in cultures and that the gap junctional communication does not appear to be directly related to the growth rates of these two cell lines in cultures. Gap junctions in both cell lines are formed by Cx43.

Comparison of structure, immunolabeling, and dye transfer

The evidence obtained from electron microscopy, immunofluorescence microscopy, as well as dye-coupling, immunoblotting, and Northern analyses clearly indicate that 9L cells form communicating gap junctions and synthesize readily detectable amounts of Cx43 mRNA and protein. In comparison, gap junctional structures in C6 glioma cells have been elusive (Tiffany-Castiglioni *et al.*, 1986; Naus *et al.*, 1993). In this study for first time ultrastructural evidence by lanthanum hydroxide impregnation has been presented for the presence of occasional small gap junctions in C6 cells. It is quite possible that gap junctions have not been detected in these cells by previous workers because they are rare. Alternatively, the appearance of gap junctions in the present study could be due to revertants in the C6 cultures; the latter might indicate that C6 cells have potential to form gap junctions. This possibility has been shown for C6 cells in cultures by Radu *et al.* (1982). In contrast, 9L cells, presumably due to greater constitutive Cx43 protein expression, readily display recognizable gap junctions in electron micrographs. In the present study, evidence for the ability of 9L cells to communicate via gap junctions has been provided by dye coupling and scrape-loading/dye transfer experiments.

No previous report on gap junction proteins in 9L cells has been published, but astrocytes from neonatal rat brain and C6 cells are known to express Cx43 (Dermietzel *et al.*, 1989; Giaume *et al.*, 1991; Naus *et al.*, 1991). Immunofluorescence microscopy showed a punctate localization of antibody at cell-cell appositions as well as perinuclear fluorescence, which is a typical distribution pattern for gap junctional protein antigens (Hertzberg, 1985). The punctate labeling on cell membranes represents the channel forming connexons.

Comparison of connexin and mRNA expression

On immunoblots, the size of the Cx43 band, and its phosphorylated form in 9L cells, correspond to the previously reported 41 and 43 kD bands of Cx43 in rat brain (Yamamoto *et al.*, 1992; Nagy *et al.*, 1992). The difference between the levels of Cx43 protein in 9L and C6 is notable as the protein could not be detected in C6 cells. The α_1S antibodies have previously been characterized (Risek *et al.*, 1992) and are known to bind to phosphorylated as well as unphosphorylated forms of Cx43. Therefore, the inability to detect the Cx43 band in C6 cells on immunoblots is possibly due to very low constitutive levels of its expression in these cells, as previously reported by other authors (Naus *et al.*, 1991; 1993). A higher molecular weight band is recognized by α1s antibodies in lanes containing 9L and C6 samples on the immunoblot (Fig. 5.8). Some membrane proteins have a tendency to aggregate, and connexins are known to undergo heat-induced aggregation in the presence of SDS (Hertzberg, 1985). Therefore, the higher band in the 9L and C6 lanes may represent a low level of aggregated connexin43. Also, Northern blot analyses indicated that the expression of the Cx43 message was higher in 9L cells than in C6 cells. Only the Cx43 was expressed in these cells, and Cx26 (another connexin found in the brain) was not detected by Northern blot. As indicated by our dye transfer results, higher levels of Cx43 expression in 9L cells correlated with greater gap junctional communication between these cells. These experiments clearly showed that intercellular communication among 9L cells was much greater than among C6 cells. A low level of intercellular communication among C6 cells is in agreement with an earlier report (Naus *et al.*, 1991).

Gap junctions are under various regulatory controls at almost all levels including transcription of mRNA to the opening and closing of intercellular channels. Presence of a transcript for a given connexin, its protein, or even the gap junction structures does not necessarily mean that the cells are communicating through gap junctions. Thus, in the light of the results obtained from the present study, it is clear that 9L rat glioma cells

express connexin43, form gap junctions, and that these gap junctions do allow intercellular passage of molecules. Therefore, 9L glioma cells are suitable for in vitro studies on gap junctions.

Antisense expression for inhibition of gap junctions

The ubiquity of gap junctions has been a drawback for the assessment of their precise role in cellular physiology. Systems in which gap junctions could be selectively inhibited have been lacking. Although a number of factors are known to cause gap junctional channel closure (Bennett *et al.*, 1991), their physiological side effects have been transitory, and not well understood.

Antibodies to gap junctions have been used in blocking gap junctional communication (see review by Goodenough and Musil, 1993). A functionally significant study of inhibition of gap junctions by injection of antibodies into Xenopus embryos has been reported by Warner *et al.* (1984). In this study the injection of connexin antibodies in precursor cells of the anterior central nervous system resulted in abnormal development of the brain, eye, and other anterior structures. Although antibodies can be effectively used to inhibit gap junctional communication, their mechanism of action, site of action, and kinetics are not understood. A rational approach to inhibit gap junctional channel formation in cultures is to use site-specific antibodies to the extracellular domains of connexin, thus preventing intermolecular recognition. This approach has not been reported yet (Goodenough and Musil, 1993).

It has been known that gap junction formation in Novikoff cells is dependent on the expression of cell adhesion molecules, probably due to facilitation of close interaction between junction-forming membranes. In a study by (Meyer *et al.*, 1992) exposure of cells to antibodies to cell adhesion molecules blocked gap junction formation between cells.

Thus, several different methods to inhibit gap junctions have been reported and applied, but these methods are not specific, may require sophisticated equipment, and may cause undesirable side effects (Larson, 1990). In a review article, Goodenough and Musil (1993) suggest a dominant negative mutation approach for the specific ablation of gap junctional communication. This approach requires construction of mutant connexin genes and their expression in targeted cells. The mutant connexin gene product would physically interact with parental connexin but would be non-functional. A better and more straightforward method of specific ablation of connexins is antisense RNA

expression (Bennett *et al.*, 1991). Antisense RNAs are known to play a role in normal gene regulation in prokaryotes. Also, in eukaryotes, the roles of antisense RNAs in normal gene regulation are becoming known (Bentley *et al.*, 1986; Hennikoff *et al.*, 1986; Williams and Fried, 1986; Kimelman and Kirschener, 1989; Tosic *et al.*, 1990). With the advent of techniques to introduce genes into mammalian cells, it is now possible to manipulate the expression of a particular gene by antisense techniques. Inhibition of genes using antisense RNA or DNA is possible, either by providing cells with exogenous antisense oligonucleotides (reviewed by Helene and Toulme, 1990; Giles and Tidd, 1992; Persaud and Jones, 1994), or by stably transfecting them with an antisense synthesizing plasmid. Experimental expression of antisense RNA using a gene transfection method has been shown to be a powerful means for genetic manipulation, and a number of studies have successfully used antisense RNA techniques (Resnicoff *et al.*, 1994; Wu *et al.*, 1994; Gutierrez *et al.*, 1994; Albert and Morris, 1994; Redekop and Naus, 1995). The stable transfection method has been used in the present study because once transfection is achieved, cells can be maintained for a longer time in cultures with selective medium.

Design for transfection method

In the present study, the aim was to stably transfect the 9L rat glioma cells with an antisense Cx43 cDNA containing a plasmid construct that could be induced to synthesize antisense RNA to Cx43. In order to select the transfected cells, another plasmid SFFV.neo (Fuhlbrigge *et al.*, 1988), containing a selectable neomycin resistance gene, was co-transfected. The ratio of the antisense to neomycin resistance carrying plasmids was 3:1. Thus, selection for the neomycin resistance would ensure higher probability of obtaining the antisense transfection in the same cell. It would also mean that for each neomycin resistant gene copy, there would be more than one copy of the antisense cDNA plasmid. The antisense cDNA for connexin43 was under the control of a mouse metallothionein promoter. This promoter activity can be induced by exposure to heavy bivalent metal ions such as Cd^{++} and Zn^{++}, and by other agents such as glucocorticoids or interferon (Kaufman, 1990).

Evidence for transfection

Transfection of 9L glioma cells was initially indicated by their resistance to G418 (geneticin) antibiotic in cultures. Although, lipofectin reagent has been found to be helpful in stable transfection of exogenous DNA into cells, its efficacy is dependent on several factors including the nature of the gene being transfected and the characteristics of

the cells. Failure to stably transfect the DNA may result in a subsequent loss of the plasmid as the cells grow in cultures. Moreover, the G418 resistance marker resided on a different plasmid than the antisense connexin gene. Therefore, successful transfection was determined by Southern blot analysis to detect the presence of exogenous connexin DNA in the transfected cells. The cDNA for connexin43 has no restriction site for Pst I. There is only one intron in the connexin43 gene (Sullivan *et al.*, 1993) which is situated in the 5'-non-translated region of the gene, therefore the Cx43 gene also lacks this site in its coding region. Thus, Pst I-digested genomic DNA samples should show only one DNA band on Southern blots when hybridized with a probe corresponding to the translated part of the cDNA. Consistent with this prediction, the untransfected 9L DNA lane in the Figure 5.2 shows only one band, whereas transfected AB5 and AC4 cells show additional bands of Cx43 DNA. Multiple bands of Cx43 DNA in transfected cells represent the presence of multiple copies of the cDNA sequence.

Antisense RNA expression and loss of connexin43 and gap junctions in AB5 cells

Expression of sense and antisense connexin43 RNA

RNA analysis is typically done by filter hybridization (Northern blots) and solution hybridization (RNase protection assays). Of these, the RNase protection assays are reported to be ten-fold more sensitive than Northern blot analyses. Although DNA probes are used for detection of RNAs on Northern blots, the determination of sense or antisense RNA is not possible by using DNA probes which are synthesized off both the strands. Ribonucleotide probes can be synthesized from cDNA cloned in vectors containing two prokaryotic promoters on either side of the cDNA insert. Thus, synthesis of ribonucleotide probes in sense or antisense directions would be possible using different promoters. Therefore, RNase protection assays with sense ribonucleotide probes were used to detect the antisense RNA in transfected AB5 samples. RNase protection assays demonstrated that antisense Cx43 RNA synthesis was induced in these cells. Expression of the antisense RNA was under the control of a mouse metallothionein promoter which has a metal responsive sequence and the induction mechanism involves a cellular factor that interacts with this sequence in the presence of bivalent metal ions (Kaufman, 1990). The lane containing riboprobes, but no RNase contained the undegraded RNA (lane A, Fig. 6.13), whereas the lanes containing RNase-treated samples showed the protected bands (lanes D-H, Fig. 6.13). Because the probe contained the sequences which were not hybridized by the antisense RNA and also nucleotides from

the vector sequence, the protected band, as expected, is shorter than the unprotected probe. Absence of protected bands in RNase treated yeast RNA and untransfected 9L RNA samples indicated that the protected bands did not arise from non-specific RNA from the cells. In addition, the RNase treated sample of synthetic antisense connexin43 RNA (lane J, Fig. 6.13) provided evidence that the protected RNA bands arose from genuine antisense RNA. In some RNase protection assays, a faint band of antisense RNA was protected in RNA samples from untreated AB5 cells. This basal level of antisense expression is attributed to the characteristic of the metallothionein promoter which has a low basal level of expression and can even be induced by the metal ions present in fetal bovine serum (Habara-Ohkubo *et al.*, 1993).

The Northern blots, however, did not show the presence of an antisense RNA band. Antisense transcripts, that are not polyadenylated or cytoplasmic or very stable, may not be included in many RNA preparations used in the analysis (Kimelman, 1992) and may lead to negative results. Low levels of antisense RNA expression or rapid degradation of antisense RNA in the cells could also be possible reasons for failure to detect the antisense RNA.

In the present study, zinc treatment caused gradual increase in the connexin43 transcript of untransfected 9L cells. In the transfected AB5 cells, however, a gradual increase was not seen. These observations indicate that the lack of gradual increase and decrease in connexin43 was due to the effects of antisense RNA, which might be acting by binding to the connexin43 mRNA and causing its degradation, and also by inhibiting translation. Although, the effect of zinc acetate on transcriptional regulation of connexins is not known, it is possible that the connexin genes are responsive to metal ion exposure. Possible mechanisms of antisense effects on RNA stability and inhibition of translation are discussed below.

Loss of connexin43, intercellular gap junctions, and dye coupling

Direct evidence of the loss of gap junctions in zinc treated AB5 cells was obtained from the immunofluorescence labeling of gap junctions with α1s antibodies, which showed that the number and size of gap junctions decreased in a time-dependent manner. By 24 hours after treatment with zinc acetate, both the number and size of gap junctions were reduced to very low counts, whereas in untransfected 9L cells the number and size of the gap junctions remained relatively unaffected. The decrease in gap junction was correlated with the concomitant decrease in the level of connexin43 protein as seen by immunoblot analysis. Thus, it is clear that zinc treatment of AB5 cells caused a decrease

in connexin43 and in gap junctions. This effect was due to the expression of antisense RNA, because a similar large decrease in gap junctions was not seen in the untransfected 9L cells. Two studies on the inhibition of gap junctions using antisense techniques have been published (Moore and Burt, 1994; Goldberg *et al.*, 1994), but these did not attempt to measure the decrease in the gap junctions by monitoring the intercellular junctions, or shown the effects on gap junctions by immunocytological methods. Thus, this study is the first report of actually demonstrating by immunofluorescence labeling the decrease of gap junctions on cell-cell interface after antisense connexin RNA expression. These observations are supported by the decrease in the level of connexin expression on immunoblots of total cell homogenates. The decrease in gap junction labeling of the cells and loss of connexin after antisense RNA expression was further reflected in the reduction of Lucifer Yellow CH fluorescent dye transfer by scrape loading/dye transfer.

Possible mechanism of antisense effect

The exact mechanism by which the antisense RNA mediates its effects is not well understood. Different mechanisms and their combinations are thought to cause the effects (for reviews see Green *et al.*, 1986; Kimelman, 1992; Krystal, 1992; Nishikura, 1992). These mechanisms could be, (a) inhibition of transcription by binding of antisense RNA to the sense DNA template, (b) destabilization of mRNA by binding to it and rendering it susceptible to endogenous RNases, (c) prevention of maturation of the transcript by antisense RNA binding, (d) blocking of mRNA transport from nucleus to cytoplasm by binding and consequent modification, and (e) inhibition of translation by binding to the sense RNA, or by destabilizing the translational machinery.

In the present study, connexin43 mRNA was not completely lost after antisense RNA induction by zinc treatment. The level of Cx43 mRNA, however, did not constantly increase over 36 hours as the RNA in untransfected 9L cells did. This absence of increase could possibly be due to binding of the antisense RNA to the connexin43 mRNA, thereby making it susceptible to RNase attack. The level of connexin43 on immunoblots, however, decreased and became undetectable after 36 hours of zinc treatment. This loss of protein, even in the presence of connexin43 mRNA, demonstrates the effect of antisense on translation. Thus, the possible mechanisms by which the antisense RNA produced its effects in the present study seem to be both by the reduction of the level of sense transcript and by affecting translation. A double stranded RNA unwindase/deaminase has been reported to be present in a number of cells, including rat astrocytes, which can modify adenine residues in RNA-RNA duplexes to inosine (see

Nishikura, 1992). Such modification in the RNA may either lead to degradation of the molecules or alter translation of the transcripts; these changes may result in loss of the protein function. Thus, a similar mechanism may also be operative in transfected AB5 cells.

Gap junctions and growth rates of rat glioma cells

Tumorigenesis is a multistep process during which the cells acquire many special characteristics. Although, uncontrolled cellular growth by itself does not lead cells to become cancerous, increased cell proliferation is one of the characteristics of neoplastic cells. A direct correlation between gap junctional intercellular communication and cell proliferation has been hypothesized (reviewed by Loewenstein and Rose, 1992). It has also been reported that C6 glioma cells transfected with Cx43 gap junction cDNA have a slower growth rate than non-transfected C6 cells *in vitro* (Zhu *et al.*, 1991; 1992) and *in vivo* (Naus et al, 1992). This has been offered as evidence to support the role of intercellular communication in the control of cellular growth (Naus *et al.*, 1992), a hypothesis originally advanced by Furshpan and Potter (1968) and supported by Loewenstein (1979).

The present study was undertaken to test the hypothesis that gap junctional intercellular communication regulates cell growth. Gap junction protein (connexin43) synthesis in transfected AB5 cells was inhibited by antisense RNA expression and growth rates were compared with untransfected 9L cells in cultures (Chapter 6). Also, two rat glioma cells 9L and C6 were compared for their gap junctional characteristics and growth rates in cultures (Chapters 4 and 5).

Despite the observed differences in the expression of gap junctions and gap junctional communication in 9L and C6 cell lines, this study indicates that both cell lines grow at similar rates. Also, the similarity in growth rates of these two cell lines has been reported previously (Korr, 1986). Therefore, gap junctions are not necessarily the primary determining factor in the regulation of the growth rates of 9L and C6 cells.

Zinc treatment caused a change in the growth rate of both the untransfected 9L cells and the transfected AB5 cells. Inhibition of gap junctions in AB5 cells by antisense expression following zinc treatment resulted in appreciable decrease in the growth rate. It has been reported that expression of functional gap junctions in non-communicating C6 glioma cells retards their growth, indicating that growth regulatory molecules may pass through gap junction channels and control the cell growth (Zhu *et al.*, 1991; Naus *et al.*,

1992). From the above observation it would be hypothesized that inhibition of functional gap junction channels would lead to increased growth of the cells. In this study, however, inhibition of gap junctions in antisense Cx43 transfected AB5 cells resulted in retardation of cell growth. In addition, transfected cells grew at markedly slower rate than their untransfected counterparts. This effect on growth is attributed to the transfection of the cells. It is possible that the transfection of cells and resulting constitutive expression of genes not required in the normal physiology of the cell may cause metabolic overload, which in turn, may result in slowed growth rates. The growth of cells in culture is dependent upon a number of growth factors and extracellular ligands (Rozengurt, 1992), some of which remain to be identified. A study using conditioned media implicates soluble growth-regulatory factors, rather than connexins *per se*, as factors controlling cell proliferation (Zhu *et al.*, 1992). The result obtained in this study that cell growth is not affected by the expression of gap junctions is consistent with another connexin antisense vector transfection study in cultured fibroblasts (Goldberg *et al.*, 1994), in which the growth rate of transfected cells (that lacked connexin43 RNA and protein) was not affected.

Although, it has been shown that the expression of transfected connexin43 gene in communication-deficient C6 rat glioma cells resulted in a retardation of growth, it is quite possible that such an effect is caused by transfection itself. Another possibility is that transfection of the cells has caused the disruption of a regulatory gene which in turn has resulted in decreased growth and further sensitivity to zinc.

The results obtained from this study show that (a) gap junctions may affect the cell growth in 9L rat glioma but are not the major growth determining factor, (b) the antisense RNA method can be used to inhibit gap junction formation, and (c) the 9L cells provide a valuable model for studying the regulation of gap junctions in astroglia. In addition, this is the first demonstration of a direct study on the effect of inhibition of connexin43 on the growth rate of a tumor cell line.

Chapter 8

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